Doc. Ref. AM1

Appl. No. 08/935,377



WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶:

C12N 15/12, 15/27, 15/55, 15/62, 9/16,
5/08, C07K 14/535, 14/47, 14/71, A61K
35/12

A1

(11) International Publication Number:

WO 97/24438

(43) International Publication Date:

10 July 1997 (10.07.97)

(21) International Application Number:

PCT/US96/20241

(22) International Filing Date:

23 December 1996 (23.12.96)

(30) Priority Data:

08/579,823

28 December 1995 (28.12.95) US

(74) Agents: STRATFORD, Carol, A. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR,

BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT,

LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG,

US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG),

Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB,

GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ,

US

(60) Parent Application or Grant

(63) Related by Continuation US

08/579,823 (CIP)

Filed on

28 December 1995 (28.12.95)

(71) Applicant (for all designated States except US): ACTIVATED CELL THERAPY, INC. [US/US]; 291 North Bernardo Avenue, Mountain View, CA 94043 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): LAUS, Reiner [DE/US]; 2813 Porto Rosa Way, San Carlos, CA 94070 (US). RUEGG, Curtis, Landon [US/US]; 3358 Brittan Avenue, San Carlos, CA 94070 (US). WU, Hongyu [US/US]; 275 Ventura Avenue #19, Palo Alto, CA 94306 (US).

CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMMUNOSTIMULATORY COMPOSITION AND METHOD

(57) Abstract

Disclosed are therapeutic compositions and methods for inducing cytotoxic T cell responses in vitro and in vivo. The therapeutic compositions consist of antigen presenting cells activated by contact with a polypeptide complex constructed by joining together a dendritic cell-binding protein and a polypeptide antigen. Also disclosed are expression vectors and systems for producing the polypeptide complexes.

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JA	V40011				

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IMMUNOSTIMULATORY COMPOSITION AND METHOD

Field of the Invention

The present invention relates to compositions and methods for stimulation of specific cellular immune responses in vivo. More specifically, the invention is concerned with elimination of tumor cells by cytotoxic T-lymphocytes (CTL) activated in vivo or in vitro by exposure to antigen presenting cells exposed to an immunostimulatory polypeptide complex.

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Background of the Invention

The immune response of the mammalian immune system is divided into two general types: humoral immunity, mediated largely by circulating antibodies, and cellular immunity mediated by various forms of T-cells. Extracellular antigens stimulate a humoral response, while intracellular antigens such as viruses, stimulate a cellular response.

The cellular immune response to virally infected cells and tumor cells is largely mediated by cytotoxic T-lymphocytes (T_c, or CTL), when they recognize foreign antigens attached to the host cell surface as part of the Major Histocompatibility Complex (MHC), and more particularly, a common form of MHC known as MHC Class I. In contrast, antigens derived from non-viral pathogens (bacteria, fungi) are generally expressed as part of an MHC Class II complex. A different subpopulation of effector T cells (cell mediated immune cells; CMI) release cytokines that activate the host cell to destroy such pathogens.

In experimental systems, tumor-antigen specific CTL are the most powerful immunological mechanism for the elimination of tumors. CTL can be induced either *in vivo* with vaccines or can be generated *in vitro* and then be re-infused into the tumor-bearing organism. The *in vivo* induction of CTL is typically accomplished by immunization with live virus or cells (Tanaka, et al., <u>J.Immunol.</u>, (1991), <u>147</u>, 3646-52, Wang, et al., <u>J.Immunol.</u>, (1995), 4685-4692, Torre-Amione, et al., <u>Proc.Natl.Acad.Sci.U.S.A.</u>, (1990), <u>87</u>, 1486-90).

Except for a few special viral proteins such as the SV-40 large T-antigen and the Hepatitis B surface antigen, injection of isolated or soluble proteins does not result in induction of CTL (Schirmbeck, et al., <u>Eur.J.Immunol.</u>, (1993), <u>23</u>, 1528-34). CTL are induced when a protein enters the class I pathway of antigen processing. To enter this pathway the protein must be present in the cytosol of the APC. There it is degraded into peptides which are then transported into the endoplasmic reticulum, where they associate with nascent HLA class I molecules. These peptides are then displayed together with the class I molecules on the cell surface and can serve as an inducer and target of class I restricted antigen-specific CTL. Physiologically, only proteins that are endogenously synthesized by an APC enter this pathway.

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Non-cellular-delivery vehicles for proteins, such as pH-sensitive liposomes, can overcome the requirement for endogenous synthesis *in vivo* (Nair, et al., <u>J.Exp.Med.</u>,(1992), <u>175</u>, 609-12, Nair, et al., <u>J.Virol.</u> (1993), <u>67</u>, 4062-9); however, these treatments are also quite toxic to the target cells.

Induction of primary HLA class I restricted CTL by pure soluble proteins in vitro has not been reported. The most common tool for ex vivo induction of primary CTL are small (8-11-mer) synthetic peptides (Stauss, et al., Proc.Natl.Acad.Sci.U.S.A. (1992), 89, 7871-5, Carbone, et al., J.Exp.Med. (1988), 167, 1767-79). These synthetic peptides associate with class I molecules on the cell surface without the requirement for endogenous processing. When presented on the surface of an appropriate APC (such as a dendritic cell) they can then induce a primary CTL response. However, frequently these CTL do not protect against challenge with pathogens that endogenously synthesize the protein from which the peptide was derived because of their low T-cell receptor avidity (Speiser, et al., J.Immunol. (1992), 149, 972-80) and because they induce reactivity with a single epitope of the target antigen.

GM-CSF is a cytokine that has pleiotropic function both in hematopoiesis as well as in immunology. GM-CSF has been shown to promote differentiation and survival of dendritic cells. GM-CSF can be used as an systemic adjuvant (Jones, et al., <u>Eur.J.Clin.Microbiol. Infect.Dis.</u> (1994), 13, S47-53).

It is well known that immunization with soluble proteins can result in a significant antibody response. However, since class II restricted antigen presentation or direct B-cell stimulation is responsible for this effect, antibody induction has no predictive value for the stimulation of class I mediated induction of CTL. Most proteins that induce antibodies in vivo fail to induce CTL.

GM-CSF fusion proteins have been shown to induce in vivo antibody responses in a lymphoma mouse model (Levy, R. and Tao, M.-H. (1993) Nature 362: 755-758; Chen, et al., Limmunol. (1995), 3105-3117). In this study, tumor idiotype fused to GM-CSF was found to be superior to the mixture of both molecules and to other conventional adjuvants for the induction of antibody responses. In contrast to other solid tumors, antibody responses are believed to be the effector mechanism for tumor protection and for tumor therapy in lymphoma.

Moreover, in vitro induction of immunity is generally much more difficult to achieve for both cellular and humoral responses. For example, viral antigen-transfected fibroblasts induce Class I restricted CTL in vivo in mice but fail to do so in vitro (Kündig, et al., Science (1995), 268, 1343-1347). Therefore, an antibody induction study with GM-CSF fusion proteins in vivo

does not imply any of its *in vitro* utilities, and is particularly unpredictive of CTL induction *in vitro* or *in vivo*.

Other methods that have been used for *in vitro* induction of primary protein-derived CTL are osmotic shock of dendritic cells and the use of pH-sensitive liposomes (Nair, et al., <u>J.Exp.</u> Med. (1992), <u>175</u>, 609-12). However, such methods have been shown to be inherently ineffective and toxic to APC's, because they disrupt cellular membranes by physical and chemical force in order to release the protein antigen into the cytoplasm.

These limitations are overcome by the discovery encompassed by the present invention. It is the discovery of the present invention that a T-cell response, and specifically, an MHC-Class I mediated T-cell response, can be stimulated by an isolated or soluble protein, when it is presented to the immune system as part of a complex with a dendritic cell binding protein, and more particularly GM-CSF. It is the further discovery of the present invention that such a response can be stimulated *in vitro*. As discussed above, *in vitro* stimulation of such a response has not previously been demonstrated using a full-length soluble antigen. The present invention provides for induction by isolated or soluble proteins of cellular immunity *in vitro* by presenting a specific antigen to an antigen presenting cell (APC), such as a dendritic cell, as part of an immunogenic fusion protein.

An important aspect of the present invention is the choice of fusion partner protein: a dendritic cell binding protein, such as granulocyte-macrophage colony stimulating protein (GM-CSF). Without relying on any particular mechanistic theory, it is believed that the protein antigen is transported over the plasma membrane of the APC in a receptor-mediated, non-disruptive fashion. It is further believed that the dendritic cell binding portion of the fusion protein serves to preserve the viability and functionality of the APC.

An additional aspect of the invention relates to the choice of target antigen. Although several tumor related antigens have been shown to serve as targets for T-cell mediated immunity in vivo, in vitro induction by isolated soluble polypeptide antigens has not been demonstrated. (Fisk, et al., J.Exp.Med. (1995), 181, 2109-2117). In experiments carried out in support of the present invention, it has now been demonstrated that tumor associated proteins not previously shown to be target antigens for CTL can become such targets by priming CTL with GM-CSF fusion derivatives in vitro.

Summary of the Invention

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In one aspect, the invention is directed to a therapeutic composition for stimulating a cellular immune response. The composition is an isolated, stimulated potent antigen presenting

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cell, such as an activated dendritic cell, that is able to activate T-cells to produce a multivalent cellular immune response against a selected antigen. In general, the measured T-cell response is substantially higher than a T-cell response produced by such potent antigen presenting cells stimulated by the selected antigen alone. In a preferred embodiment of the invention, the potent antigen presenting cell is stimulated by exposing the cell in vitro to a polypeptide complex that consists essentially of a dendritic cell-binding protein and a polypeptide antigen. Preferably, the polypeptide antigen is either a tissue-specific tumor antigen or an oncogene gene product. However, it is appreciated that other antigens, such as viral antigens can be used in such combination to produce immunostimulatory responses.

In another preferred embodiment, the dendritic cell-binding protein that forms part of the immunostimulatory polypeptide complex is GM-CSF. In a further preferred embodiment, the polypeptide antigen that forms part of the complex is the tumor-specific antigen prostatic acid phosphatase. In still further preferred embodiments, the polypeptide antigen may be any one of the oncogene product peptide antigens Her2, p21RAS, and p53. The polypeptide complex may also contain, between the dendritic cell-binding protein and the polypeptide antigen, a linker peptide.

In a related aspect, the invention includes a method of activating an isolated antigen presenting cell in vitro. According to the method, the activation includes contacting an isolated antigen presenting cell with a polypeptide complex. The polypeptide complex used in this 20 method is as described above; that is, it consists essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen, in any of the embodiments described above. According to the method, the activated antigen presenting cell is effective to activate a T-cell to produce a multivalent cellular immune response that is substantially higher than that produced by antigen presenting cells contacted with the selected polypeptide antigen alone. In a preferred embodiment, the antigen presenting cell is a dendritic cell, isolated as described herein.

In yet another related aspect, the invention includes a method of inducing a cytotoxic T-cell response in a vertebrate subject. According to this aspect of the invention an isolated dendritic cell is contacted with an immunostimulatory polypeptide complex according to any of the embodiments described above for a period of time effective to activate the antigen presenting cell. The antigen presenting cell is then injected into the mammalian subject. In a preferred embodiment, the antigen presenting cell that is activated and injected is a dendritic cell.

In further related aspects, the invention also includes the polypeptide complexes formed as described above. As described above, such polypeptide complexes are preferably formed

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from a dendritic cell binding protein, preferably GM-CSF, and a polypeptide antigen. The polypeptide antigen is preferably a tissue-specific tumor antigen such as prostatic acid phosphatase (PAP), or an oncogene product, such as Her2, p21RAS, and p53; however, other embodiments, such as viral antigen antigens, are also within the contemplation of the invention.

In further related aspects, the invention also includes expression vectors and expression systems for producing the above-described immunostimulatory fusion proteins, as well as substantially purified nucleic acid molecules that encode such fusion proteins. In preferred embodiments, the nucleic acid molecules code for fusion proteins consisting essentially of GM-CSF and prostatic acid phosphatase or of GM-CSF and Her2.

The invention also includes a novel prostate carcinoma cell line, HLA A2.1 cells, that can be used a target cell for testing tumor cell killing, as described herein.

Brief Description of the Figures

- FIG. 1 shows nucleic acid (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of PAP-GM-CSF fusion protein having as a peptide linker gly-ser resulting from the Bam HI linker (underlined);
 - FIG. 2 shows the amino acid sequence of the fusion protein Human Prostatic Acid Phosphatase/Human GM-CSF, with the PAP signal sequence not present in the mature protein shown in small caps, potential N-glycosylation sites marked "C", and potential disulfide bridges marked "S-S";
 - FIGS. 3A and 3B show schematic representations of PAP-GM expression vectors pCEP4 PAPGM (3A) and PAPHGM-BAC (3B) used in mammalian (293 cells) and insect (SF21) cell lines, respectively;
- FIG. 4 shows a graph of GM-CSF bioactivity of mammalian and baculovirus-derived PAP-25 GM-CSF fusion proteins;
 - FIG. 5 shows a graph of acid phosphatase bioactivity of PAP-GM-CSF fusion proteins;
 - FIG. 6 shows a graph of lysis of prostate carcinoma cells by CTL primed with PAP-GM-CSF and stimulated with PAP-GM-CSF pulsed antigen presenting cells;
- FIG. 7 shows a bar graph depicting blockade by HLA-class I blocking antibody of lysis of prostate carcinoma cells by PAP-GM-CSF primed CTL;
 - FIG. 8 shows the nucleic acid (SEQ ID NO: 3) and amino acid (SEQ ID NO: 4) sequences of a GM-CSF-Her2 fusion protein in accordance with the present invention;
 - FIG. 9 shows titration of GM-CSF activity in crude Baculovirus Insect Cell (BVIC) culture supernatants from ratPAP or ratPAP-mouseGM-CSF infected Sf21 cultures as compared to

recombinant mouseGM-CSF and resultant proliferative response of rodent GM-CSF-dependent cell line GM-NFS-60;

- FIG. 10 shows an immunization scheme of COP rats with purified dendritic cells pulsed with ratPAP-ratGM-CSF;
- FIG. 11 shows nucleic acid and deduced protein sequence of the p53-GM-CSF fusion gene, where the Xba I linker that codes for serine and arginine is boxed; and
 - FIG. 12 shows the amino acid sequence of p53-GM-CSF fusion protein with the synthetic serine-arginine linker printed in boldface and underlined.

10 Detailed Description of the Invention

I. Definitions

As used herein, the term "tissue-specific antigen" refers to an antigen that is characteristic of a tissue type, including specific tumor tissues. An example of a tissue-specific antigen expressed by a tumor tissue is the antigen prostatic acid phosphatase, which is present in over 90% of all prostate tumors. By way of contrast, B-cell lymphomas produce immunoglobulin antigens that are particular to the individual tumor. Such particular tumor antigens are not considered to fall within the definition of the term "tissue-specific antigen."

The term "oncogene product" refers to any protein coded for by a gene associated with cellular transformation. Examples of oncogene products include, for example, Her2, p21RAS, 20—and-p53.

"Antigen presenting cells" (APC) are cells that are capable of activating T cells, and include, but are not limited to, certain macrophages, B cells and dendritic cells.

"Potent antigen presenting (PAP) cells" are cells which, after being pulsed with an antigen, can activate naive CD8+ cytotoxic T-lymphocytes (CTL) in a primary immune response.

The term "dendritic cell" refers to any member of a diverse population of morphologically similar cell types found in lymphoid or non-lymphoid tissues. These cells are characterized by their distinctive morphology, high levels of surface MHC-class II expression (Steinman, et al., Ann. Rev. Immunol. 9: 271 (1991); incorporated herein by reference for its description of such cells). These cells can be isolated from a number of tissue sources, and conveniently, from peripheral blood, as described herein.

The term "dendritic cell binding protein" refers to any protein for which receptors are expressed on a dendritic cell. Examples include GM-CSF, IL-1, TNF, IL-4, CD40L, CTLA4, CD28, and FLT-3 ligand.

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II. Immunostimulatory Polypeptide Complexes

A. Selection of Components of the Polypeptide Complex

An immunogenic polypeptide formed in accordance with the present invention is generally characterized as an isolated polypeptide antigen which is covalently linked to a dendritic cell-binding protein.

1. Polypeptide Antigens

As stated above, isolated polypeptide antigens do not generally stimulate activation of T-cells in vivo or in vitro. It is the discovery of the present invention that certain types of polypeptide antigens, when coupled to a dendritic cell-binding proteins, such as those discussed below, stimulate T-cell activation.

The present invention identifies as particularly useful in this capacity (1) tissue-specific tumor antigens and (2) oncogene product peptide antigens. In the context of the present invention, the term "tissue-specific tumor antigens" refers to antigens that are common to specific tumor types. By way of contrast, antigens that are specific to a particular individual tumor, such as the B cell lymphoma tumor-associated idiotype antigen, are distinguishable from tissue-specific tumor antigens in that they have a characteristic epitope that varies from individual to individual. Such antigens are less useful in the context of the present invention, since a immunostimulatory reagents must be tailored to the individual tumor, and consequently do not form part of the invention.

Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and GP 100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER-2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma. Some of these antigens (CEA, HER-2, CD 19, CD20, idiotype) have been used as targets for passive immunotherapy with monoclonal antibodies with limited success.

Thus, examples of tissue-specific tumor antigens include, but are not limited to prostatic acid phosphatase (PAP; associated with prostatic tumors), Melan-A/MART-1 (associated with melanoma; Coulie et al., 1994, <u>J.Exp.Med.</u> 180:35, Hawakami et al., 1994, <u>PNAS</u> 91:3515,

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Bakker et al., 1994, <u>J.Exp.Med.</u> 179:1005), tyrosinase/albino (associated with melanoma; Kawakami et al., 1994, <u>J.Exp.Med.</u>), CD19, CD20 and CD37 (associated with lymphoma).

Likewise, oncogene product peptide antigens have been identified that are common to specific tumor types. These polypeptides can be incorporated into polypeptide complexes of the present invention as reagents that can be used generally to stimulate T-cell responses effective to react with tumors bearing such antigens. Oncogene product peptide antigens include but are not limited to HER-2/neu (Beckmann et al., 1992, Eur.J.Cancer 28:322) associated with human breast and gynecological cancers, and carcinoembryonic antigen (CEA) associated with cancer of the pancreas.

A variety of tumor markers are known in the art or are commercially available and include, but are not limited to the tissue-specific antigens that include cytokeratins, prostate-specific antigen (Wang et al., 1977), gp75/brown (Brichard et al., 1993, J.Exp.Med. 178:489) associated with melanoma, melanotransferrin (Real et al., 1984, J.Exp.Med. 160:1219), MUC1 (Barnd, 1989, PNAS USA 86:7159 and Vijayasaradhi et al., 1990, J.Exp.Med. 171:1375) associated with pancreas and breast cancer; oncogene/tumor suppressor genes that include EGF-R (Osborne et al., 1980), estrogen receptor, progesterone receptor, retinoblastoma gene product, myc associated with lung cancer, ras, p53, nonmutant associated with breast tumors, MAGE-1,3 (van der Bruggen et al., 1991, Science 254:1643 and Gaugler et al., 1994, J.Exp. Med. 179:921) associated with melanoma, lung, and other cancers.

Isolated viral antigens may include HIV antigens gp120, gp41, gag, RT, NEF, VIF; influenza antigens HA, core and matrix,; EBV antigens: EBNA, BFLF1, BOLF1, BGLF2, LMP2a, LMP2b, BBRF1, BBRF2, and P11L27; and human papilloma virus.

Polypeptide antigens such as those described above can be isolated, synthesized or recombinantly expressed according to methods known in the art. In most cases, DNA coding sequences have been identified for these molecules. In addition, many of the so-called "tumor markers" are available commercially. Such isolated antigens can be complexed with a dendritic cell binding protein chemically, as discussed below, or fusion protein constructs may be produced recombinantly, according to methods well known in the art.

As an example of the foregoing, prostatic acid phosphatase (PAP) is the prostate-specific isozyme of the ubiquitous enzyme acid phosphatase. PAP is a secreted molecule that has been identified as a serum tumor marker that is specific for prostate cancer. (Vihko, et al., <u>FEBS Lett.</u> (1988), 236, 275-281, Solin, et al., <u>Biochem.Biophys.Acta</u> (1990), <u>1048</u>, 72-77). There is no evidence from the literature that PAP by itself might serve as an inducer and target of CTL. As is demonstrated below, the present invention shows that PAP can serve both as an

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inducer of CTL and as a target in prostate cancer cells, when combined with the dendritic cell binding protein GM-CSF and used to stimulate antigen presenting cells (exemplified by dendritic cells) that are then used to prime CTL.

2. Dendritic cell binding proteins

The second component of the polypeptide complex of the present invention is a dendritic cell binding protein. As mentioned above, without relying on any particular mechanistic theory, it is believed that the presence of such a molecule in covalent complex with a protein antigen facilitates transport of the antigen over the plasma membrane of the antigen presenting cell, and more particularly, the dendritic cell, in a receptor-mediated, non-disruptive way. It is further believed that the dendritic cell binding portion of the fusion protein serves to preserve the viability and functionality of the APC.

An example of a dendritic cell binding protein is granulocyte-macrophage colony stimulating factor (GM-CSF). This glycoprotein, which has an apparent molecular weight of about 23-33,000 by SDS-PAGE, is a cytokine that has pleiotropic function both in hematopoiesis as well as in immunology. Both human and murine GM-CSF are synthesized with a 17-amino acid hydrophobic leader sequence that is proteolytically cleaved during secretion. The mature proteins are 127 (human) or 124 (murine) amino acids, and have core polypeptide molecular weights of 14,700 and 14,400, respectively, but share only 52% amino acid identity. The factor has been found to play a stimulatory role in the differentiation and survival of dendritic cells and is active in both glycosylated and de-glycosylated forms.

Human and murine GM-CSF have been shown to bind to both high affinity ($K_D=20$ -60 pM) and low affinity ($K_D=1$ -6 nM) binding sites on cells of the monocyte-macrophage, neutrophil and eosinophil cell lineages. Competition for binding by another member of the hemopoietic colony stimulating factors, Multi-CSF, has been shown when the binding is carried out at 37°. Binding of GM-CSF to high affinity receptors results in rapid internalization and degradation of GM-CSF (Metcalf, D. and Nicola, N.A. (1995) The Hemopoietic Colony-Stimulating Factors, Cambridge University Press, NY.). These properties may be used to serve as a guide to the selection of additional dendritic cell binding proteins useful in forming immunostimulatory polypeptide complexes in accordance with the present invention.

B. Formation of Polypeptide Complexes

Polypeptide complexes can be formed by chemical means, such as by conventional coupling techniques known in the art. For example, the peptides can be coupled using a dehydrating agent such as dicyclohexylcarbodiimide (DCCI) to form a peptide bond between the two peptides. Alternatively, linkages may be formed through sulfhydryl groups, epsilon

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amino groups, carboxyl groups or other reactive groups present in the polypeptides, using commercially available reagents. (Pierce Co., Rockford, IL).

Polypeptide complexes can also be formed recombinantly as fusion proteins according to methods known in the art. Example 1 details the methods used to produce a GM-CSF-PAP fusion protein in accord with the present invention. Briefly, human PAP was cloned from a prostate carcinoma cell line according to methods known in the art. The stop codon at the 3' end of the sequence was mutated away, and a Bam HI site inserted in its place, to fuse the PAP cDNA to GM-CSF DNA. GM-CSF DNA was cloned from a PBMNC library according to standard methods. A Bam HI site was inserted at the 5' end of the DNA, and an XbaI cloning site was inserted at the 3' end, along with an in-frame stop codon. PCR-generated cDNA's were digested with appropriate restriction enzymes and cloned into restriction vectors for transfection into specific mammalian or insect cell lines. FIG. 1 shows nucleic acid and deduced amino acid sequences of the PAP-GM-CSF fusion polypeptide having a gly-ser peptide linker. FIG. 2 further illustrates the fusion protein sequence with potential glycosylation sites indicated as "C" and probable disulfide bridges shown as "S-S." FIGS. 3A and 3B show schematic representations of the PAP-GM-CSF expression vectors used for transfecting mammalian (293) and insect (SF21) cell lines, respectively.

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The fusion expression vectors were used to transfect COS cells (transient expression) as well as mammalian 293-EBNA cells (Invitrogen) and insect SF21 cells (Clontech, Palo Alto, 20—CA). Fusion protein products were recovered from the tissue culture supernatants, and affinity purified by passage over an anti-human PAP monoclonal antibody immunoaffinity column. Analysis by SDS-PAGE revealed protein bands migrating at 75 kD and 64 kD as products from mammalian and insect cells, respectively. The 75 Kd band corresponds to a size that is approximately 19.5 Kd larger than the predicted size of the PAP-GM-CSF polypeptide backbone which is 55.5 Kd. This can be explained by the presence of 5 potential N-glycosylation sites in the sequence, glycosylation at which would increase the apparent M, of the protein, and is consistent with the fact that 293-EBNA cells contain fully functional human glycosylation machinery. The insect cell-derived fusion protein was approximately 8.5 Kd larger than the PAP-GM-CSF peptide backbone. These data are consistent with the known glycosylation patterns in Si21 cells, which are reported to utilize N-glycosylation sites but which only add truncated carbohydrates that typically end with the addition of a single mannose residue.

The fusion molecules were tested for PAP and GM-CSF bioactivities in appropriate assays, detailed in Example 2. Both insect and mammalian cell-derived fusion proteins exhibited GM-

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CSF activity, as evidenced by their ability to support growth of GM-CSF dependent cell lines (FIG. 4). Similarly, both products exhibited PAP activity (FIG. 5).

Fusion proteins constructed to incorporate oncogene product antigens are exemplified by incorporation of the oncogene product Her2. Her2 is a growth factor receptor belonging to the EGF-R family of receptors. It is over-expressed by breast cancer cells, ovarian cancer cells and a variety of other cancer cells. The cDNA coding for the extracellular domain of Her2 is cloned from a breast cancer cell line and fused to the GM-CSF cDNA, essentially as detailed for PAP-GM-CSF, above. Production of the soluble protein can be verified using Her2-specific monoclonal antibodies in an ELISA test, according to methods well-known in the art. The fusion protein includes the sequences for the extracellular domain (amino acids 1-652) of Her2 (GenBank) and GM-CSF (FIG. 8). In this particular fusion protein the two proteins are linked by a leucine/glutamic acid linker which is generated by inserting a XHO I site.

The cellular tumor suppressor gene p53 is cloned and fused to GM-CSF, as described in Example 7. The sequence of the recombinant fusion gene is shown in FIG. 11, and the sequence of the produced polypeptide p53-GM-CSF is shown in FIG. 12. The p53-GM-CSF fusion protein is used to generate anti-p53 immunity as described for the induction of anti-PAP immunity by PAP-GM-CSF in Examples 3, 4 and 6.

Other oncogene product antigens are similarly incorporated into fusion proteins according to the methods described herein, using published sequences. In addition, other antigens, such as viral antigens, may be part of a fusion construct, according to the methods described herein.

While the foregoing description describes particular embodiments of the present invention, it will be appreciated that persons skilled in the art can substitute different antigens, vectors and expression cell lines, according to known methods, to prepare immunostimulatory polypeptide complex compositions in accordance with the principles described above. In addition, it is appreciated that the invention may also be practiced by inserting between the dendritic cell binding protein and the polypeptide antigen, a linker peptide or protein such as ubiquitin, according to recombinant methods known in the art.

III. Stimulation of T-cells

An important aspect of the present invention is the utility of the above-described polypeptide complex constructions in a method to target the antigen protein partner to Antigen Presenting Cells (APC), such as the cell type known as the "dendritic cell", described above. In accordance with the invention, the targeting occurs in a manner that results in entry into the class I pathway of antigen processing. The APC is then used to prime CTL ex vivo and in vivo, according to the methods discussed below.

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Dendritic cells are highly potent APC's, and are the only APC that can prime naive CTL. While dendritic cell precursors present in human blood can take up antigen, they do not function as potent APC's. On the other hand, the mature dendritic cell is the most potent APC, but it does not take up antigen spontaneously *in vitro*. In the past, it was necessary to treat mature dendritic cells with physical force (liposomes, osmotic shock) or to coat them with exogenous small (8-11 amino acids in length; generally 9-mer) peptide antigens to enable them to act as APC.

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The present invention enables introduction of an exogenously added protein into the class I pathway of a mature dendritic cell. Such induction can be effected *in vitro*, by isolating APC's such as dendritic cells, "pulsing" or contacting them with the polypeptide complex for an extended period of time, then using the pulsed APC's to stimulate autologous T-cells *in vitro* or *in vivo*. In the latter case, the pulsed APC's are administered (approximately 10⁷ cells/injection) to the subject. The response of the subject is measured by monitoring the induction of a cytolytic T-cell response, a helper T-cell response and antibody response towards the antigen in peripheral blood mononuclear cells by methods well known in the art. Multiple doses may be administered to produce an adequate response.

The use of GM-CSF fusion antigen-stimulated dendritic cells yields superior results to other approaches, such as the peptide pulsed dendritic cell preparations. That is, while dendritic cells pulsed with 8-11-mer peptides are able to induce immunity, such immunity is directed to a single T-cell epitope. Proteins incorporated into liposomes or delivered to the dendritic cells by osmotic shock induce reactivity towards multiple T-cell epitopes; however, this process is relatively ineffective due to the inherent toxicity of these treatments to dendritic cells.

In contrast, GM-CSF fusion antigens of the present invention induce immunity towards multiple epitopes and preserve and enhance at the same time viability and function of the dendritic cell. In practice, the compositions of the present invention are found to induce a cellular (T-cell) activation that is multivalent and substantially higher than that produced by a selected antigen alone.

In experiments carried out in support of the present invention, the fusion protein consisting of PAP and GM-CSF described in the previous section was used for *in vitro* introduction to dendritic cells and subsequent activation of cytolytic T cells, as detailed in Example 4. Briefly, HLA-A2.1 positive PBMNC were isolated by standard methods and primed with the fusion protein for 2-5 days. The cell mixture was depleted of CD4⁺ T-cells, separated into high and low density fractions, and the separate cultures were re-stimulated weekly with autologous PAP

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GM-CSF pulsed APC's. Lytic potential of the T-cells present in the fractions was assessed using a standard chromium release assay using an HLA-A2.1-transgenic prostate carcinoma cell line as target. This novel cell line was constructed according to the methods detailed in Example 3 herein and is useful in screening and analysis of HLA class I restricted cytotoxic T-lymphocytes.

Results of the lysis assays are presented in FIG. 6. As shown, of the four different T-cell cultures tested, three displayed substantial dose-dependent cytotoxicity towards the prostate carcinoma target. The highest degree of cytotoxicity was effected by cells that fractionated into the high density pellet fraction on day 5 (open circles, 5-day P). High (open squares, 2-day P) and low (closed squares, 2-day IF) density cells primed for two days showed roughly equal potency. Cell cultures derived from day 5 low density interface fraction (closed circles, 5-day IF) displayed little or no cytotoxicity.

FIG. 7 shows that the tumor-specific cytolysis was substantially reduced in the presence of the HLA class I-specific blocking antibody W6/32 at an Effector: Target (E/T) ratio of 10:1 and is completely eliminated by the antibody at an E/T ration of 3.3/1. Control antibody CA 141 did not reduce T-cell mediated killing. These experiments demonstrate that the interaction with the target cell is mediated by via classical T-cell receptor/HLA-class I restricted antigen-specific pathway.

The foregoing results demonstrate the efficacy of fusion polypeptide complexes formed in accordance with the present invention in stimulating T-cell responses in vitro. These responses can be compared to those stimulated by the antigen alone (in the absence of the dendritic cell binding protein). In addition, their multivalent character can be tested by standard methods. IV. Therapeutic applications

A. In vitro/ex vivo Therapy

The present invention provides for induction by isolated or soluble proteins of cellular immunity *in vitro* by presenting a specific antigen to an antigen presenting cell (APC), such as a dendritic cell, as part of an immunogenic fusion protein. As discussed above such induction is not generally observed *in vitro* using soluble, whole antigens as induction materials.

In practice, dendritic cells are isolated from an individual, using known methods, or preferably, as described in Example 5. The dendritic cells (or other APC's) are mixed with 10 ng/ml equivalent of GM-CSF fusion antigen, as described in Example 4. The cell preparation may then be depleted of CD4⁺ T-cells by solid phase immunoadsorption and further fractionated to enrich for cells having cytolytic activity. Doses of about 10⁷ cells are then administered to the subject by intravenous or central injection according to established

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procedures (e.g., infusion over 30 to 60 minutes). The responsiveness of the subject to this treatment is measured by monitoring the induction of a cytolytic T-cell response, a helper T-cell response and antibody response towards the antigen in peripheral blood mononuclear cells by methods well known in the art.

In addition to the direct in vivo administration regiment described above, the APC cells can be used, for example, in ex vivo somatic therapy, in vivo implantable devices and ex vivo extra-corporeal devices. They can also be employed in the screening of antigenicity and immunogenicity of peptide epitopes from tumor- and virus-specific antigens.

In certain cases, it may be advantageous to use cells obtained from one individual to treat a condition in a second individual. For example, HIV-infected individuals with AIDS are often not able to mount anti-viral T-cell responses. In such cases, CTL can be isolated from healthy HLA-matched individuals, such as siblings, be stimulated or primed with antigen-pulsed DC in vitro, expanded, and administered back to the HIV-infected individuals.

B. In vivo Therapy

The fusion protein compositions described herein can also be administered directly to an individual as a vaccine, in order to stimulate the individual's cellular immunity pathways in vivo. Here a dose of about 5 to 200 microgram/kg fusion protein, is administered, preferably at days 0, 14 and 28 with an optional boosting dose at 6 months. The response of the subject is measured by monitoring the induction of a cytolytic T-cell response, a helper T-cell response and antibody response towards the antigen in peripheral blood mononuclear cells by methods well known in the art.

C. Results from Experimental Models of Immunostimulation

Example 6A provides details of the construction of fusion antigens and injection protocols used in an experimental rat model of immunostimulation. A fusion protein made up of rat PAP and rat GM-CSF was injected directly into rats (in vivo stimulation) or to stimulate rat dendritic cells for later injection into rats (ex vivo induction). It is necessary to use rat molecules and cells, since human effector cells do not function normally in other species and are, in addition, antigenic in other species, such as rat. Moreover, the target antigen (PAP) is different between humans and other species, and human GM-CSF would not be expected to produce the same type of effect in other species. Nonetheless, it is understood that the use of rat cells in rats should provide a model system for the use of human cells and proteins in humans.

As detailed in Example 6, rat prostatic acid phosphatase (ratPAP) was prepared recombinantly and was fused to rat GM-CSF. Dendritic cells from rat splenic tissue were

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pulsed with ratPAP-ratGM-CSF and were injected into normal rats. In addition, alternate delivery systems were tested, since autologous PAP-directed immunization has not previously been carried out, to the best of our knowledge.

In studies carried out in support of the present invention and detailed in Example 6B, rats were immunized with either (i) ratPAP-ratGM-CSF pulsed dendritic cells, (ii) ratPAP-ratGM-CSF alone, or (iii) rat PAP in conventional CFA adjuvant. Rat organs (prostate plus six major organ systems) were later examined for immunopathology evoked by autologous PAP-directed immunization. According to the rat model system, it was expected that a successful immune response would result in immune targeting of PAP-expressing organs, and in particular, the prostate.

As shown in Table 1, the histopathology studies show that immunizations with rat PAP-rat GM-CSF pulsed dendritic cells or with rat PAP rat GM-CSF fusion protein induce organspecific autoimmunity that is limited to the prostate. In contrast, immunization with ratPAP/CFA protein did not produce this result. This result demonstrates that PAP-GM-CSF itself and PAP-GM-CSF pulsed dendritic cells produce antigen-specific autoimmunity that is significantly better than, and in fact, cannot be produced by, the antigen alone in association with a conventional adjuvants.

Table 1 Organ Histopathology of immune and non-immune rats

20	Rat No.b	Brain	Lung	Heart	Liver	Kidney	Colon	Prostate	Comments, Prostate
	1C	N	N	N	N	N	N	N	N .
	2C	N	N	Z	N	N	N	N	N
	3C	N	N	N	И	N	N	N	N
25	4C	N	N	N	N	N	N	N	N
	5T	N	N	N	Z	N	N		Moderate subacute multifocal interstitial prostatitis
	6T	N	N	N	N	N	N		Mild scattered inflammation in the interstitium
	7 T	N	N	N	N	N	N		Trace to scattered inflammation in the interstitium
	8T	N	N	N	N	N	N		Mild multifocal inflammation in the interstitium
30	1 CFA	ND	ND	ND	ND	ND	ND	N	
	2 CFA	ND	ND	ND	ND	ND	ND	N	

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1 PAP GM	ND	ND	ND	ND	ND	ND	1	Mild multifocal lymphocytic inflammation in the interstitium
2 PAP GM	ND	ND	ND	ND	ND	ND		Mild multifocal lymphocytic inflammation in the interstitium

* Pathology grading: ND = Not evaluated; N = No significant lesions; 1 = trace/mild; 2 = mild; 3 = moderate; 4 = severe.

b Immunization groups: animals 1C, 2C, 3C, 4C were non-immunized controls; animals 5T, 6T, 7T, 8T were immunized with rat PAP rat GM-CSF pulsed dendritic cells; animals 1CFA, 2CFA were immunized with rat PAP immersed in CFA; animals 1 PAP GM, 2 PAP GM were immunized with recombinant rat PAP rat GM-CSF.

The following examples illustrate, but in no way are intended to limit the invention.

Examp

Example 1 Construction of PAP/GM-CSF Fusion Proteins

If not described otherwise, general cloning procedures were performed according to standard techniques as described in Sambrook, et al., <u>Molecular Cloning: A Laboratory Manual</u> (1989).

Human PAP was cloned from the prostate carcinoma cell line LnCaP.FGC (American Type Culture Collection, Rockland Maryland; "ATCC"). Synthetic oligonucleotide primers containing the sequence CGGCTCTCCTCAACATGAGAGC were custom synthesized according to standard methods by Keystone Labs (Menlo Park, CA). These primers are homologous to the 5° end of the known PAP cDNA sequence which is published in the GenBank database.

Hind III, Mun I or Xho I restriction sites were attached, according to the requirements of the particular expression vector used. On the 3' end an oligonucleotide of the sequence CACAGGATCCATCTGTACTGTCCTCAGTACC was constructed that mutated away the stop codon 387 of the PAP sequence and substituted for it a Bam HI site that codes for amino acids glycine and serine. This Bam HI site was used to fuse the PAP cDNA to the GM-CSF cDNA, which was cloned from peripheral blood mononuclear cells (PBMNC), based on the sequence which is published in GenBank. At the GM-CSF 5' end a Bam HI site was attached to an oligonucleotide GACTGGATCCGCACCCGCCCGCTCGCCC that is homologous to the nucleotides that code for amino acids 18-23 in the GM-CSF sequence. The 3' end of GM-CSF was generated with a an oligonucleotide GATCTCTAGAGCTTGGCCAGCCTCATCTGG that ends after the in frame stop of GM-CSF and creates an Xba I cloning site. Poly A+ RNA was isolated from cell line LnCaP.FGC and from PBMNC with the Micro Fast track kit (Invitrogen) according to the manual supplied by the manufacturer. The Poly A+ RNA was

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then reverse transcribed with the cDNA cycle kit (Invitrogen) according to procedures described in the accompanying manual. First strand cDNA was then subjected to 25 cycles of polymerase chain reaction (PCR) with the above described primers. The conditions on a Perkin-Elmer thermal cycler 9600 were as follows. 25 cycles of amplification were performed at: Denaturation: 94°C, 15 seconds; annealing 55°C, 15 seconds; extension 72°C, 60 seconds. The 25 cycles were followed by a final extension period of 420 seconds at 72°C. The PCR products were analyzed on agarose gels. They were digested with the appropriate restriction enzymes and cloned into the vectors pCR3 (Invitrogen) to create pCR3-PAP-GM (not shown), pCEP 4 (Invitrogen) to create pCEP4-PAP GM (FIG. 3A) and into pBacPac 8 (Clontech) to create PAPHGMBAC (FIG. 3B). The DNA sequences of the cloned constructs were confirmed using standard methods on a fluorescent sequencer Model ABI 373A (Applied Biosystems, Foster City, CA). The nucleotide sequence and the deduced amino acid sequences are presented as SEQ ID NO: 1 and SEQ ID NO: 2, in FIG. 1, respectively.

pCR3 PAP-GM was electroporated into COS-7 cells (ATCC) for transient expression experiments. After it was confirmed that a protein of the predicted size, immunological identity and function could be expressed transiently in COS-7 and 293-EBNA (Invitrogen) cells stable transfectants were generated in the human embryonic kidney cell line 293-EBNA, using an episomal expression vector pCEP4 (Invitrogen, San Diego, CA). After electroporation and selection in hygromycin, recombinant clones were generated by plating the cells under limiting dilution conditions and screening for PAP bioactivity in the cellular tissue culture supernatants. The highest producing clones were adapted to protein-free media and grown in CellMax hollow fiber bioreactors (Gibco, Gaithersberg, MD). Spent media from the cultures were collected, pooled and clarified by centrifugation. They were then passaged over an immunoaffinity column that was made by coupling the human PAP-specific monoclonal antibody ATCC HB8526 (ATCC) to a Sepharose resin. After washing, the bound material was eluted at low pH, neutralized and dialyzed against physiological buffer. The eluted fraction was analyzed by denaturing SDS-PAGE electrophoresis under reducing conditions. The resulting gel showed a single protein band at 75 kD which corresponds to the predicted size of fully glycosylated PAP-GM-CSF.

PAPHGM-BAC was also used to generate a recombinant Autographa californica nuclear polyhedrosis virus (AcNPV, baculovirus) by homologous recombination of PAPHGM-BAC with BacPAK6 viral DNA (Clontech, Palo Alto, CA). Reagents were used from the BacPAK baculovirus expression system (Clontech) and procedures were carried out essentially as described in the product manual. PAPHGM-BAC and BacPAK6 were co-transfected into SF21

cells (Clontech) by lipofection. The spent tissue culture supernatant was collected on day 5. It was titered onto fresh SF21 cells which were then grown in semisolid media for another 4 days. After the monolayers were stained with neutral red, viral plaques were identified and picked with a Pasteur pipet. Recombinant plaque-purified virus was eluted into fresh media and was then used to screen for production of PAP-GM-CSF in fresh SF21 cells. Positive plaques were identified and used to generate viral stocks and recombinant protein in subsequent rounds of infecting fresh SF21 cells. The media of production cultures were collected three days after infection. They were then processed as described for PAP-GM-CSF that was derived from 293-EBNA cells. Analysis of the immunoaffinity-purified protein revealed a single protein band at 64 kD after silver staining of an SDS-PAGE gel.

Example 2 Bioactivity of PAP-GM-CSF fusion proteins

PAP-GM-CSF fusion proteins from all expression systems described in Example 1 were analyzed for their ability to support the growth of GM-CSF dependent cell lines. They were also analyzed for enzymatic activity in acid phosphatase assays. Standard bioassays were used to determine the GM-CSF bioactivity.

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GM-CSF Activity. The GM-CSF dependent human erythroleukemia cell line TF-1 (ATCC, Rockville, MD) and the acute monocytic leukemia cell line AML-193 (ATCC) were used to analyze whether GM-CSF retains its bioactivity after fusion to PAP. The cell lines which are routinely cultured in GM-CSF-containing media were starved in regular media for 24 hours before the assay. They were plated at 1500 cells per well in triplicates in tissue culture medium. Test supernatants or recombinant GM-CSF as a positive control were added to the cells. Cells were cultured for 72 hours and were then pulsed for 4 hours with 1 microcurie tritiated thymidine per well to determine rate of DNA synthesis. FIG. 4 shows that both mammalian derived and insect cell-derived PAP-GM-CSF support the growth of GM-CSF dependent cell lines. The calculated relative bioactivity of PAP-GM-CSF is 20% of the activity of control recombinant GM-CSF dependent cell lines on a molar basis.

Acid Phosphatase Activity. The bioactivity of the second component of the fusion protein was determined in an enzymatic assay for acid phosphatase activity. Acid phosphatase was measured as the ability of the protein to hydrolyze para-nitrophenyl phosphate (pNPP) at acid pH. Briefly, the test liquid was diluted in 50mM sodium citrate pH 4.8. pNPP is added to a final concentration of 2 mg/ml. After 30 minutes incubation at 37°C, an equal volume of 1M NaOH was added to the reaction. Hydrolyzed pNPP under these conditions has a yellow color which can be quantified with a spectrophotometer at 405 nm. FIG. 5 shows that both

mammalian derived and insect cell derived PAP-GM-CSF hydrolyzed pNPP under acid conditions.

Thus, it is clear that the original biological activity of both PAP and of GM-CSF is conserved in PAP-GM-CSF fusion proteins.

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Example 3 Generation of a target cell line for HLA class I-restricted cytotoxic T-lymphocytes

If not otherwise described, all tissue culture techniques, cell manipulations and assays were performed according to standard techniques.

In order to generate a prostate cancer cell line that could be used as a target cell on a defined genetic background an HLA A2.1-transgenic prostate cancer cell line was generated. HLA A2.1 is the best studied restriction element in human HLA class I restricted immune responses and is the most frequent allele in Caucasians. A cDNA that codes for the published sequence (GenBank) of HLA A2.1 was isolated and cloned from the lymphoblastoid cell line JY. HLA A2.1 heavy chain cDNA was amplified with the sense primer 5-AgACgCCgAggATggCC-3' and the anti-sense primer 5-CCTCTCTggAACAggAAAgATg-3'. Methods and conditions were as described for PAP and GM-CSF except that the cell line JY (obtained from Dr. Ed Engleman, Stanford University Blood Bank, Stanford, CA) was used as starting material. The resulting gene fragment was cloned into the pCR3 vector with the TA-cloning kit (Invitrogen). The prostate carcinoma cell line LnCaP.FGC (ATCC) was transfected with this expression plasmid which confers the expression of HLA A2.1. The parent cell line does not express the A2.1 allele. After drug selection in G418 (Gibco) the resulting transfectants were selected for HLA A2.1 expression by solid phase immunoadsorption ("panning") with a HLA A2.1-specific monoclonal antibody (BB7.1, ATCC). The resulting cell line homogeneously expressed HLA-A2.1 whilst its parent remained negative. This novel transgenic cell line is uniquely useful in the screening and analysis of HLA class I restricted cytotoxic T-lymphocytes.

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Example 4 Induction of prostate cancer-specific CTL by PAP-GM-CSF

A T-cell in vitro priming and expansion system was used to establish the utility of PAP-GM-CSF in the generation of HLA class I restricted CTL.

35 HLA-A2.1-positive PBMNC were isolated by standard methods on density gradient (FICOLL-HYPAQUE, Pharmacia Fine Chemicals, Piscataway, NJ) having a density of 1.077 gr/ml. The cells were primed with 10 ng/ml equivalent of PAP-GM-CSF for two or five days.

(GM-CSF potency equivalent was measured on a GM-CSF dependent cell line as detailed in Example 3; the actual weight used was twenty-fold higher because of the different size and specific activity of PAP-GM-CSF.) The cell preparation was then depleted of CD4+ T-cells by solid phase immunoadsorption and separated into low density and high density cells over a 1.068 gr/ml density gradient. The different fractions were then cultured separately in AIM V media (Gibco, Gaithersberg, MD) supplemented with rIL-2 (20 U/ml). Autologous PBMNC that were cultured in 20 ng/ml PAP-GM-CSF in Aim V media were used as antigen presenting cells for re-stimulation at weekly intervals. Lytic potential of the cells was assessed in a standard 4-hour chromium release assay with the HLA-A2-1-transgenic prostate carcinoma cell line LnCaP.FGC as a target.

FIG. 6 shows induction of Prostate-carcinoma LnCaP.FGC/A2.1-specific cytotoxic T-lymphocytes by PAP-GM-CSF pulsed antigen presenting cells. HLA-A2.1 positive PBL were primed with 10 ng/ml GM-CSF equivalents of PAP-GM-CSF for two or five days. The cultures were depleted of CD4⁺ T-cells and separated into low (IF) and high (P) density fractions over a Nycodenz density gradient having a density of 1.068 gr/ml.

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To investigate whether the observed cytotoxicity was a HLA-class I-restricted CD8+cytolytic T-cell mediated phenomenon a blocking assay with the monomorphic HLA class I-specific monoclonal antibody W6/32 (ATCC) antibody was performed. W6/32 blocks HLA class I mediated killing in standard assays, whilst control antibody CA141 is specific for HLA class II (DR) and will not interfere with class I restricted killing. T-cell cultures which were derived from the 5 day pellet fraction (described above) which displayed the highest cytotoxicity were used for this experiment. The T-cell lines used in experiment contained 38% CD3/CD8 positive T-cells. Their lytic potential was assessed in a standard 4-hour chromium release assay with the HLA-A2-1-transgenic Prostate carcinoma cell line LnCaP.FGC as a target. FIG. 7 shows that tumor-specific cytolysis was substantially reduced in the presence of the HLA class I blocking antibody W6/32 at an Effector:Target (E/T) ratio of 10:1 and is completely eliminated by the antibody at an E/T ration of 3.3/1. Control antibody CA 141 did not reduce T-cell mediated killing. These experiments demonstrate that the interaction with the target cell is mediated by via classical T-cell receptor/HLA-class I restricted antigen-specific pathway.

Example 5 Preparation of Dendritic cells

Buffy coats prepared from one unit of blood from HLA-A0201 positive volunteer healthy donors are obtained from the Stanford University Blood Center (Stanford, CA). Cells are

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harvested from the leukopacs, diluted to 60 mL using Ca⁺⁺/Mg⁺⁺ free phosphate buffered saline (D-PBS; Gibco Laboratories, Grand Island, NY) and layered over two 15 mL columns of organosilanized colloidal silica (OCS) separation medium (prepared as described by Dorn in U.S. Patent 4,927,749, incorporated herein by reference, at a density 1.0720 gr/ml, pH 7.4, 280 mOsm/kg H₂O) in 50 mL centrifuge tubes, preferably cell-trap tubes. The OCS medium is preferably prepared by reacting and thus blocking the silanol groups of colloidal silica (approx. 10-20 nm diameter particles) with an alkyl trimethoxy silane reagent.

Related colloidal silicas and methods for production thereof are disclosed in U.S. Patent 4,927,749 to Dorn. In a preferred embodiment, the OCS density gradient material is diluted to an appropriate specific density in a physiological salt solution supplemented with polyvinyl-pyrrolidone (PVP) such as PVP-10 available from Sigma Chemical Co. (St. Louis, MO).

The tubes are centrifuged at $1000 \times g$ for 35 minutes at room temperature. The centrifuge run is allowed to stop without braking and the peripheral blood mononuclear cells (PBMC), present at the interface, are harvested.

PBMC are re-suspended in D-PBS, centrifuged once at $650 \times g$ for 10 minutes and twice more at $200 \times g$ for 5 minutes to remove platelets. Platelet-depleted PBMC are re-suspended in 60 mL of D-PBS, layered on top of two columns of 15 mL of OCS (density 1.0610 gr/ml, 280 mOsm/kg H_2O) in a centrifuge tube and centrifuged at $650 \times g$ for 25 minutes at 4°C without braking. The resulting interface (primarily monocytes) and pellet cells (primarily lymphocytes) are harvested and washed with D-PBS by centrifugation at room temperature (once at $650 \times g$ for 10 minutes and twice thereafter at $200 \times g$ for 5 minutes).

In instances where the dendritic cells are used to generate peptide-specific cytotoxic T lymphocytes (CTL) for purposes of elucidating their antigen presentation function, the interface fraction (mostly monocytes) is re-suspended in cold pooled human AB serum (Irvine Scientific, Santa Ana, CA) to which an equal volume of 80% AB serum 20% dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO) is added dropwise. The resulting cell suspension is aliquoted into cryovials and frozen in liquid nitrogen. The monocytes can be used for restimulation of CTL for expansion.

The pellet fraction is re-suspended in 100 mL of AB Culture Medium, inoculated into two T-75 tissue culture flasks and cultured in a humidified 5% CO_2 incubator for 40 hours. Following the incubation, the non-adherent cells are harvested by moderate pipeting, washed and re-suspended at a concentration of $2 - 5 \times 10^6$ cells/mL in AB Culture Medium. The cell suspension is layered onto four columns of 4.0 mL OCS separation medium (density 1.0565)

gr/ml, pH 7.4, 280 mOsm/kg H_2O), in AB Culture Medium and centrifuged at 650 \times g for 20 minutes at room temperature without braking.

The interface and pellet cells are harvested and washed in AB Culture Medium (Basal RPMI-1640 medium, Gibco Laboratories, Grand Island, NY) by centrifugation once at 650×10^{-5} g for 10 minutes and twice thereafter at 200×10^{-5} g for 5 minutes each at room temperature. The yield and viability of both cell fractions is estimated by counting on a hemocytometer using trypan blue exclusion.

The purity of dendritic cells in the interface fraction is quantified following analysis on a flow cytometer (FACS). Dendritic cells are characterized as negative for cell phenotype markers CD3 (T lymphocytes), CD14 (monocytes), CD16 (NK cells) and CD20 (B-cells) and positive for HLA class II expression using dual staining with HLA-DR (on the FITC channel) and a cocktail of CD3, CD14, CD16, CD20 (on the PE channel). Dual staining with IgG2a on both the FITC and PE channels can be used as isotype control.

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The morphology of the cells can also be evaluated using photomicroscopy. The DC enriched fraction contains large sized veiled cells with cytoplasmic processes extending from the cell surface, features characteristic of DC.

Example 6 In vivo efficacy of PAP-GM-CSF-pulsed dendritic cells

20 A. Construction, expression and bioactivity of recombinant ratPAP and GM-CSF fusion

Recombinant rat PAP-rat-GM-CSF was constructed as follows: rPAP cDNA was amplified from first strand cDNA made from mRNA isolated from rat prostrate (Harlan) using primers which delineate the fragment containing nucleotides 15-1177 (Genbank Acc. M32397) and which add an exogenous Xho I restriction site at the 5'-end and exogenous BamHI and Bln I sites at the 3'-end to facilitate insertion into the pBacPAK8 vector. In the absence of GM fusion, the Bln I site encodes an in-frame stop codon. Mature rat GM-CSF cDNA was amplified by PCR from first strand cDNA made from mRNA isolated from ConA-stimulated rat splenocytes using primers which delineate nucleotides 1-384 (GenBank Acc. U00620) and add an exogenous BamHI site at the 5'-end and an exogenous Xba I site at the 3'-end.

The ratPAP and ratPAP-ratGM-CSF plasmids were each mixed with linearized BV viral genome plasmid and the mixtures were transfected into SF21 cells using Lipofectin as supplied in a recombinant BV transfection kit (Clontech). Six days after transfection, the culture supernatants were collected and titrated on Sf21 monolayers under agarose to form viral plaques. Four days later the cells were stained with neutral red and candidate viral plaques

were picked and expanded on Sf21 cells to screen for recombinant BV using PAP enzymatic activity as a readout. PAP+ BV clones were chosen and expanded in SF21 large-scale suspension cultures for viral stocks and subsequently for protein production using protein-free Sf900 II media (Gibco/BRL).

All proteins exhibited PAP enzymatic activity as shown by hydrolysis of PNPP, the enzyme substrate used in a standard acid phosphatase assay and, in the case of GM-CSF fusion proteins, GM-CSF bioactivity as shown by bioassay on GM-NFS-60 cells. GM-CSF bioactivity of a representative fusion protein example, ratPAP-ratGM-CSF (FIG. 9), was determined in a GM-CSF rat cell proliferation assay carried out using the GM-CSF-dependent cell line GM-NFS-60. Five thousand cells were plated per well in 200 μ l of media with the designated amounts of cytokine. Proliferation was determined by pulsing with 1 μ Ci [3 H]thymidine per well during the final 4 hr of a 48 hr assay. Crude baculovirus culture supernatant or with 10ng/ml commercially available recombinant mouse GM-CSF (rmGM) was diluted and added to the cultured cells as indicated, and proliferation was measured.

RatPAP was expressed as a fusion protein with a tail of 6 histidine-residues (ratPAP(His₆)) attached to its C-terminus. It was purified by means of metal-chelate affinity chromatography (Ni-NTA columns), according to standard methods. A typical purification resulted in >90% pure recombinant protein. Purification of ratPAP-ratGM-CSF was performed by a combination of ion-exchange/hydrophobic interaction chromatography. This procedure typically yields >50% pure rat PAP-GM-CSF.

B. Immunization of Animals

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Male inbred rats were used in all studies that were designed to address the immunogenicity of ratPAP in vivo. The goal of such studies was to determine whether immunity towards the autoantigen rat PAP could be elicited.

Normal 8-week old male rats (Wistar or COP) were immunized with PAP in CFA (approx. $200\mu g$) or with PAP-GM-CSF fusion protein ($200\mu g$). After conclusion of the immunization, the rats were euthanized and their prostates were examined histologically. An additional group of rats was treated with ratPAP-ratGM-CSF pulsed dendritic cells. Dendritic cells from rat spleens were isolated as outlined in FIG. 10, pulsed overnight with ratPAP-ratGM-CSF that had been purified from baculovirus culture supernatant by a combination of cation-exchange chromatography, hydrophobic interaction chromatography and anion-exchange chromatography. Cells (5-10 x 10^6 cells/rat) were injected back into syngeneic hosts. Two weeks after the third immunization, animals were sacrificed and prostate, brain, lungs, heart, liver, kidney and colon

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were analyzed by a veterinarian histopathologist and compared to tissues from control animals which were not immunized.

Phenotype of rat dendritic cells was determined using flow cytometry. To determine their antigen-presenting capacity, cells were used as stimulators in an allogeneic mixed lymphocyte reaction and their allostimulatory capacity to total splenocytes and to adherent splenocytes was compared. The typical range for dendritic cell purities obtained by this method is generally between 30% and 80%. The antigen-presenting capacity of dendritic cells from the rats was analyzed by using them as stimulators in an allogeneic mixed lymphocyte reaction with SD rat splenocytes as responders. Their allostimulatory capacity was compared to total splenocytes and to adherent splenocytes from both COP and SD strains.

SD-splenocytes were not stimulated by syngeneic SD-splenocytes, adherent splenocytes or dendritic cells. When allogeneic COP stimulators are used, the dendritic cell fraction stimulates several orders of magnitude stronger than spleen cell or adherent spleen cells from COP rats.

Histopathology of immunized rats. Histopathology was performed by a consulting veterinary histopathologist according to standard procedures. Animals were surveyed for histopathological changes to prostate, brain, lungs, heart, liver, kidney and colon, as summarized in Table 1. All organs in all control animals (1C, 2C, 3C, 4C) showed no significant change. All organs except the prostates in the treated animals showed no significant change. All animals treated with rat PAP rat GM-CSF pulsed dendritic cells suffered from interstitial prostatitis. The intensity varied: one animal (7T) had grade 1 (= trace) lesions, two animals (6T, 8T) had grade two lesions (= mild) and one animal (5T) had grade 3 (= moderate) lesions. Animals which were immunized with rat PAP in CFA (1CFA,2CFA) did not show any histopathology of the prostate. Animals that were immunized with rat PAP rat GM-CSF protein (1 PAP GM, 2 PAP GM) had a grade 1 interstitial lymphocytic prostatitis.

Example 7 Construction and expression of p53-GM CSF fusion proteins

The cellular tumor suppressor gene p53 was cloned according to publicly known sequences (Genbank database release 91, entry HSP53) by means of polymerase chain reaction with the sense primer CgCggATCCTCACTgCCATggAggAgC and the antisense primer CTAgTCTAgA-CTCTgAgTCAggCCCTTCTgTC which includes an XBA I site that codes for a serine-arginine linker. GM-CSF was cloned as described in Example 1 except that primers CTAgTCTAgATC-TgCACCCgCCCgCTCgCCC(sense) and CCggAATTCTCAgTgATggTgATggTgATgCgATC-CTCTCATCTCCTggACTggCTCCCAgC (anti-sense) were used. The antisense primer codes

for the sequences MetArgGlySerHisHisHisHisHisHisHis which is attached to the C-terminus of GM-CSF and can be used for detection and metal-chelate affinity chromatography purification of the fusion protein. The resulting sequences are shown in FIGS. 11 and 12. Recombinant baculovirus transfer vector and recombinant baculovirus was generated as described in Example 1. When expressed in SF21 insect cells as detailed in Example 1 this baculovirus produces p53-GM-CSF fusion protein which is used to generate anti-p53 immunity as described for the induction of anti-PAP immunity by PAP-GM-CSF in Examples 3, 4 and 6. The sequence of the recombinant fusion gene is shown in FIG. 11. The sequence of the produced polypeptide p53-GM-CSF is shown in FIG. 12.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

- 1. A therapeutic composition, comprising an isolated potent antigen presenting cell effective to activate T-cells to produce a multivalent cellular immune response against a selected antigen, at a T-cell activation level substantially higher than that produced by such a potent antigen presenting cells stimulated by the selected antigen alone.
- The therapeutic composition of claim 1, wherein the potent antigen presenting cell is stimulated by exposing the cell in vitro to a polypeptide complex consisting essentially of a dendritic
 cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene gene product.
 - 3. The therapeutic composition of claim 2, wherein the dendritic cell-binding protein is GM-CSF.

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- 4. The therapeutic composition of claim 2, wherein the polypeptide antigen is the tumor-specific antigen prostatic acid phosphatase.
- 5. The therapeutic composition of claim 2, wherein the polypeptide antigen is an oncogene product peptide antigen selected from the group consisting of Her2, p21RAS, and p53.
 - 6. The therapeutic composition of claim 2, wherein the polypeptide complex further comprises, between said dendritic cell-binding protein and said polypeptide antigen, a linker peptide.

- 7. The therapeutic composition of claim 2, wherein the potent antigen presenting cell is an activated dendritic cell.
- 8. A method of activating an isolated antigen presenting cell in vitro, comprising

 contacting said isolated antigen presenting cell with a polypeptide complex consisting essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen,

wherein said activated antigen presenting cell is effective to activate a T-cell to produce a multivalent cellular immune response that is substantially higher than that produced by antigen presenting cells contacted with the selected polypeptide antigen alone.

- 5 9. The method of claim 8, wherein said dendritic cell-binding protein is GM-CSF.
 - 10. The method of claim 8, wherein said polypeptide antigen is the tissue-specific tumor antigen prostatic acid phosphatase.
- 10 11. The method of claim 8, wherein the polypeptide complex is a fusion protein.
 - 12. The method of claim 8, wherein said isolated antigen presenting cell is a dendritic cell.
- 13. A method of inducing a cytotoxic T-cell response in a vertebrate subject, comprising

 contacting an isolated antigen presenting cell with a polypeptide complex comprising a

 dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group

 consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen for a

 period of time effective to activate said antigen presenting cell, and

injecting said antigen presenting cell into the mammalian subject.

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- 14. The method of claim 13, wherein said antigen presenting cell is a dendritic cell.
- 15. A polypeptide complex, comprising an isolated tissue-specific tumor antigen covalently linked to a dendritic cell-binding protein.

- 16. The polypeptide complex of claim 15, wherein said dendritic cell-binding protein is GM-CSF.
- 17. The polypeptide complex of claim 15, wherein said tissue-specific tumor antigen is prostatic acid phosphatase.
 - 18. The polypeptide complex of claim 15, wherein the complex is a fusion protein.

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- 19. The polypeptide fusion protein complex of claim 15, which further includes, between said dendritic cell binding protein and said tissue-specific tumor antigen, a linker peptide.
- 20. A polypeptide complex, comprising an isolated oncogene product peptide antigen covalently linked to a dendritic cell-binding protein.
 - 21. The polypeptide complex of claim 20, wherein said dendritic cell-binding protein is GM-CSF.
- 10 22. The polypeptide complex of claim 20, wherein said oncogene product peptide antigen is selected from the group consisting of Her2, p21RAS, and p53.
 - 23. The polypeptide complex of claim 20, wherein the complex is a fusion protein.
- 15 24. The polypeptide fusion protein of claim 20, which further includes, between said dendritic cell binding protein and said oncogene product antigen, a linker peptide.
- 25. An expression vector for producing an immunostimulatory fusion protein, comprising a nucleic acid molecule encoding a polypeptide complex consisting essentially of a dendritic cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen, said nucleic acid molecule inserted into an expression vector, wherein said nucleic acid molecule is operably linked to a selected promoter able to initiate transcription in a selected host cell.
- 26. The expression vector of claim 25, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the tissue-specific tumor antigen prostatic acid phosphatase.

- 27. The expression vector of claim 25, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the oncogene product peptide antigen Her2.
- 28. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and prostatic acid phosphatase.

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- 29. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and Her2.
- 30. An expression system for producing a fusion protein consisting essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen, comprising
 - a nucleic acid sequence encoding a dendritic cell-binding protein,
- a nucleic acid sequence encoding the polypeptide antigen, each of said nucleic acid sequences inserted into an expression vector, wherein said nucleic acid sequences are operably linked to a promoter able to initiate transcription in a selected host cell, and

said expression vector is carried within the host cell.

1/10

CGGCTCTCCT CAACATGAGA GCTGCACCCC TCCTCCTGGC CAGGGCAGCA	50
MetArg AlaAlaProL euLeuLeuAl aArgAlaAla	
AGCCTTAGCC TTGGCTTCTT GTTTCTGCTT TTTTTCTGGC TAGACCGAAG	100
SerLeuSerL euGlyPheLe uPheLeuLeu PhePheTrpL euAspArgSe	
TGTACTAGCC AAGGAGTTGA AGTTTGTGAC TTTGGTGTTT CGGCATGGAG	150
rValLeuAla LysGluLeuL ysPheValTh rLeuValPhe ArgHisGlyA	
ACCGAAGTCC CATTGACACC TTTCCCACTG ACCCCATAAA GGAATCCTCA	200
spArgSerPr oIleAspThr PheProThrA spProIleLy sGluSerSer	
TGGCCACAAG GATTTGGCCA ACTCACCCAG CTGGGCATGG AGCAGCATTA	250
TrpProGlnG lyPheGlyGl nLeuThrGln LeuGlyMetG luGlnHisTy	
TGAACTTGGA GAGTATATAA GAAAGAGATA TAGAAAATTC TTGAATGAGT	300
rGluLeuGly GluTyrIleA rgLysArgTy rArgLysPhe LeuAsnGluS	
CCTATAAACA TGAACAGGTT TATATTCGAA GCACAGACGT TGACCGGACT	350
erTyrLysHi sGluGlnVal TyrIleArgS erThrAspVa lAspArgThr	
TTGATGAGTG CTATGACAAA CCTGGCAGCC CTGTTTCCCC CAGAAGGTGT	400
LeuMetSerA laMetThrAs nLeuAlaAla LeuPheProP roGluGlyVa	
CAGCATCTGG AATCCTATCC TACTCTGGCA GCCCATCCCG GTGCACACAG	450
lSerIleTrp AsnProIleL euLeuTrpGl nProIlePro ValHisThrV	
TTCCTCTTTC TGAAGATCAG TTGCTATACC TGCCTTTCAG GAACTGCCCT	500
alProLeuSe rGluAspGln LeuLeuTyrL euProPheAr gAsnCysPro	
CGTTTTCAAG AACTTGAGAG TGAGACTTTG AAATCAGAGG AATTCCAGAA	550
ArgPheGlnG luLeuGluSe rGluThrLeu LysSerGluG luPheGlnLy	
GAGGCTGCAC CCTTATAAGG ATTTTATAGC TACCTTGGGA AAACTTTCAG	600
sArgLeuHis ProTyrLysA spPheIleAl aThrLeuGly LysLeuSerG	
GATTACATGG CCAGGACCTT TTTGGAATTT GGAGTAAAGT CTACGACCCT	650
lyLeuHisGl yGlnAspLeu PheGlyIleT rpSerLysVa lTyrAspPro	
TTATATTGTG AGAGTGTTCA CAATTTCACT TTACCCTCCT GGGCCACTGA	700
LeuTyrCysG luSerValHi sAsnPheThr LeuProSerT rpAlaThrGl	-
GGACACCATG ACTAAGTTGA GAGAATTGTC AGAATTGTCC CTCCTGTCCC	750
uAspThrMet ThrLysLeuA rgGluLeuSe rGluLeuSer LeuLeuSerL	
TCTATGGAAT TCACAAGCAG AAAGAGAAAT CTAGGCTCCA AGGGGGTGTC	800
euTyrGlyIl eHisLysGln LysGluLysS erArgLeuGl nGlyGlyVal	
CTGGTCAATG AAATCCTCAA TCACATGAAG AGAGCAACTC AGATACCAAG	850
LeuValAsnG luIleLeuAs nHisMetLys ArgAlaThrG lnIleProSe	
CTACAAAAA CTTATCATGT ATTCTGCGCA TGACACTACT GTGAGTGGCC	900
rTyrLysLys LeuIleMetT yrSerAlaHi sAspThrThr ValSerGlyL	
TACAGATGGC GCTAGATGTT TACAACGGAC TCCTTCCTCC CTATGCTTCT	950
euGlnMetAl aLeuAspVal TyrAsnGlyL euLeuProPr oTyrAlaSer	
TGCCACTTGA CGGAATTGTA CTTTGAGAAG GGGGAGTACT TTGTGGAGAT	1000
CysHisLeuT hrGluLeuTy rPheGluLys GlyGluTyrP heValGluMe	
GTACTATCGG AATGAGACGC AGCACGAGCC GTATCCCCTC ATGCTACCTG	1050
tTyrTyrArg AsnGluThrG lnHisGluPr oTyrProLeu MetLeuProG	

Fig. 1A

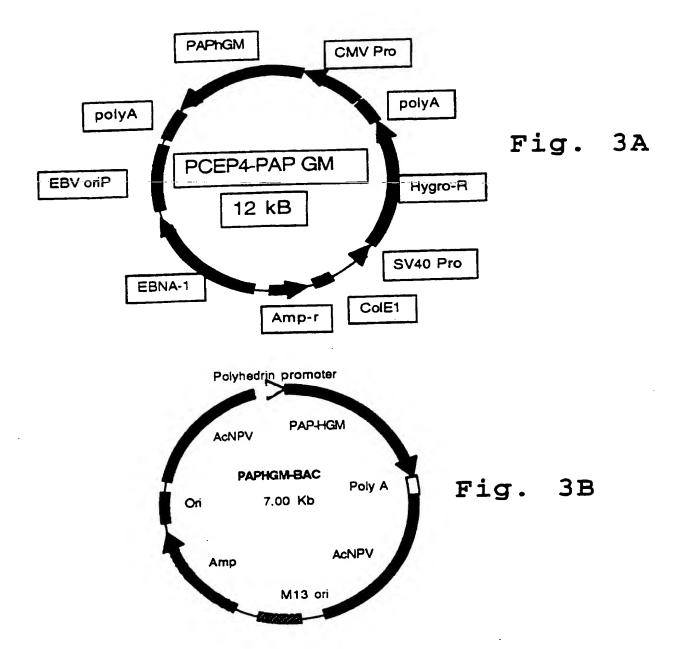
1a/10

GCTGCAGCCC	TAGCTGTCCT	CTGGAGAGGT	TTGCTGAGCT	GGTTGGCCCT	1100
lyCysSerPr	oSerCysPro	LeuGluArgP	heAlaGluLe	uValGlyPro	
GTGATCCCTC	AAGACTGGTC	CACGGAGTGT	ATGACCACAA	ACAGCCATCA	1150
VallleProG	lnAspTrpSe	rThrGluCys	MetThrThrA	snSerHisGl	
AGGTACTGAG	GACAGTACAG	ATGGATCCGC	ACCCGCCCGC	TCGCCCAGCC	1200
nGlyThrGlu	AspSerThrA	sp GlySerAl	aProAlaAro	g SerProSerP	
CCAGCACACA	GCCCTGGGAG	CATGTGAATG	CCATCCAGGA	GGCCCGGCGT	1250
roSerThrGl	${\tt nProTrpGlu}$	HisValAsnA	laIleGlnGl	uAlaArgArg	
CTCCTGAACC	TGAGTAGAGA	CACTGCTGCT	GAGATGAATG	AAACAGTAGA	1300
LeuLeuAsnL	euSerArgAs	${\tt pThrAlaAla}$	${\tt GluMetAsnG}$	luThrValGl	
AGTCATCTCA	GAAATGTTTG	ACCTCCAGGA	GCCGACCTGC	CTACAGACCC	1350
uValIleSer	${\tt GluMetPheA}$	spLeuGlnGl	${\tt uProThrCys}$	LeuGlnThrA	
GCCTGGAGCT	GTACAAGCAG	GGCCTGCGGG	GCAGCCTCAC	CAAGCTCAAG	1400
rgLeuGluLe	${\tt uTyrLysGln}$	${\tt GlyLeuArgG}$	${\tt lySerLeuTh}$	rLysLeuLys	-
GGCCCCTTGA	CCATGATGGC	CAGCCACTAC	AAACAGCACT	GCCCTCCAAC	1450
GlyProLeuT	hrMetMetAl	${\tt aSerHisTyr}$	LysGlnHisC	ysProProTh	
CCCGGAAACT	TCCTGTGCAA	CCCAGATTAT	CACCTTTGAA	AGTTTCAAAG	1500
rProGluThr	SerCysAlaT	hrGlnIleIl	${\tt eThrPheGlu}$	SerPheLysG	
AGAACCTGAA	GGACTTTCTG	CTTGTCATCC	CCTTTGACTG	CTGGGAGCCA	1550
luAsnLeuLy	sAspPheLeu	LeuValIleP	${\tt roPheAspCy}$	sTrpGluPro	
GTCCAGGAGT	GAGACCGGCC	AGATGAGGCT	GGCCAAGC		1588
ValGlnGlu.					

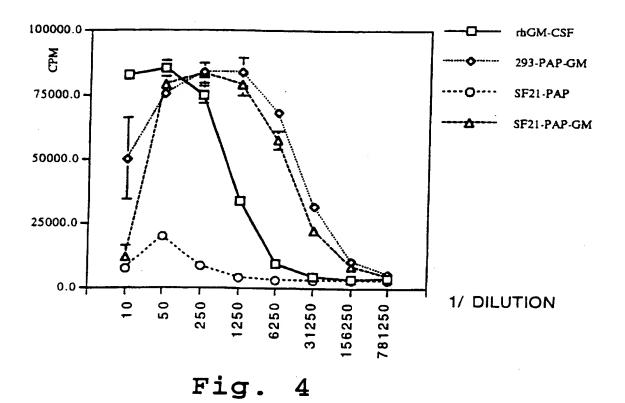
Fig. 1B

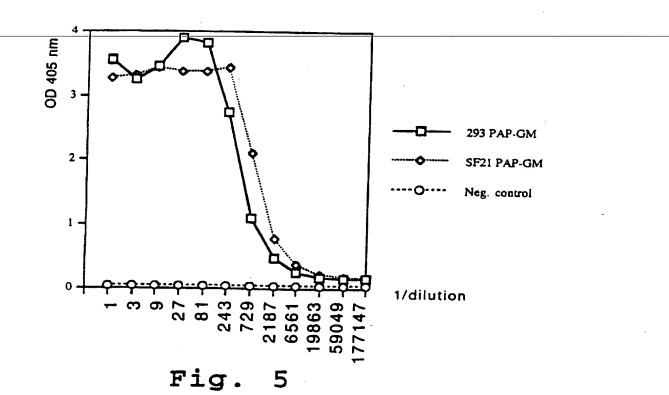
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DIFPIDPIKE SSWPQGFGQL TQLGMEQHYE LGEYIRKRYR KFLNESYKHE	100
QVYIRSIDVD RILMSAMINL AALFPPEGVS IWNPILLWQP IPVHIVPLSE	150
DQLLYLPFRN CPRFQELESE TLKSEEFQKR LHPYKDFIAT LGKLSGLHGQ	200
DLFGIWSKVY DPLYCESVHN FTLPSWATED TMTKLRELSE LSLLSLYGIH	250
KOKEKSRLOG GVLYNEILNH MKRATQIPSY KKLIMYSAHD TIVSGLOMAL	300
DVYNGLLPPY ASCELTELYF EKGEYFVEMY YRNETOHEPY PLMLPGCSPS	-3 50
CPLERFAELV GPVIPQDWST ECMTINSHQG TEDSTDGSAP ARSPSPSTQP	400
WEHVNAIQEA RRIINISRDI AAEMNETVEV ISEMFDLQEP TCLQTRLELY	4 50
KQGLRGSLTK LKGPLIMMAS HYKQHCPPIP EISCATQIIT FESFKENLKD	500
FLLVIPFDCW EPVQE	515

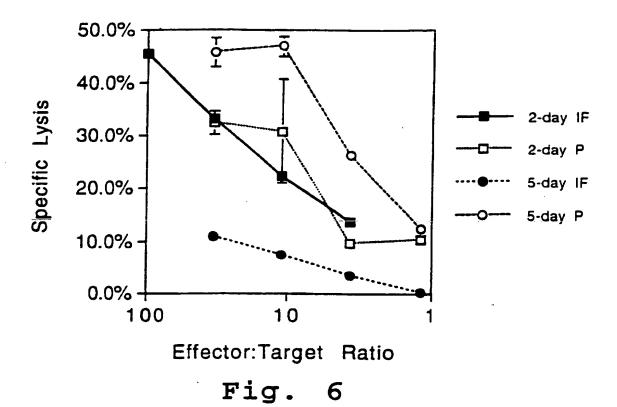
Fig. 2



חיוכחסכום. זיים מזייים







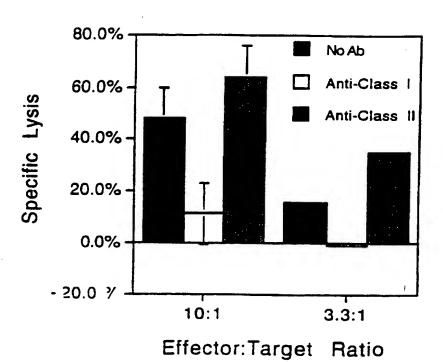


Fig. 7

AGGICAGO COSCOTTOTO COSCOTTOTO CONTROL ALEXANDER DEPORTAL ALAS MEGILLAN I BALALLAND ACTION CONTROL AND		AGTGAGCACC	ATGGAGCTGG	CCCCTTCTC	CCGCTGGGGG	CTCCTCCTCG	CCCTCTTGCC	CCCCGGAGCC	GCGAG	75
CACCAAGTG TECACCGCC AGACATGAA GCTGCGCCTC CCTGCCAGTC CCAGACCCA CCTGGACATG CTCCC Throfthy Inspiretty slewappies prohibes prohibes and control co			MetGluLeuA	laAlaLeuCy	sArgTrpGly	LeuLeuLeuA	laLeuLeuPr	oProGlyAla	AlaSe	
THINGINVAI CysThroly? hrasphetly sleukargleu ProAlaser? rociumints is eleasphet Leukar CCACCTCAC CAGGCCCA ASTOCCAC ASTOCCAC CATOCCAC GRATICCAC GRATICCACS GRATICCACS (ASTOCCAC) ASTOCCACO (ASTOCCAC) CATOCCAC CATOCCAC GRATICCACS ASTOCCACO (ASTOCCAC) ACCACTCACA ACCACCCAC		CACCCAAGTG	TGCACCGGCA	CAGACATGAA	GCTGCGGCTC	CCTGCCAGTC	CCGAGACCCA	CCTGGACATO	CTCCG	150
CCACCTCTAC CAGGGCTGC AGTGGTGCA GGGAACCTG GACCTCACC ACTGCCCAC CATCCCAGC GRISSIBLEUTY: GINGLY-VSG INVALVAGIO INGIVABLEU GILLEUTHT YTLEUPTOTH TARNALSEY LEUSE CTTCCTCAGA GATATCCAG AGTGCAGGC CACCTCT CACCAGCAGT CTTGAGGCCAC CACCACT CTTCAGGGCC ATTGTGCAGG GACACCCAGT CTTGAGGCCAGT CTCCACAGGA GACCCCAGT ACCCACCCTG TCACCACGT CTCCACGGA GACCCCCAG AGCCCCAGA ACCCCAGT ACCCACCCTG TCACCACGAG ACCCCCAGT ACCCACCCAGT CTCCACCAGA ACCCACCCCAGT CTCCCACAGA ACCCACCCCAGT CTCCCACAGA ACCCACCCCAGT CTCCCACAGA ACCCACCCCAGT CTCCCACAGA ACCCACCCCAGT CTCCCACCAGA ACCCACCCCAGT CTCCCACAGA ACCACACCAC		rThrGlnVal	CysThrGlyT	hrAspMetLy	sLeuArgLeu	ProAlaSerP	roGluThrHi	sLeuAspMet	LeuAr	
GHISLOUTY GINGLYONG INVAIVAGE NGIVACAGE CACCACTOR ACCAMENTAGE GARATCAGE GARTGACAGE CACCACTOR ATTOCCTOCA ACCAMENTAGE GARGACCA ACCAMENTAGE CACCACTOR APPLICAGE ANTOTOCCAGE GARCCACCAGET CATTOGAGAC ANTOTOCCAGE ACTOTOCAGE CACCACCAGET CATTOGAGAC ANTOTOCCAGE ACTOTOCAGE GACCCAGET CACAGACAGA GACCACCAGET CACAGACACAGE TOTOCAGAGACAT ACCACCCCTE TACAGAGGAC CACCAGACAACAA ACCACCACCTE TACAGAGGAC CACCAGGACAACAAT ACCACCACCTE TACAGAGGAC CACCAGACAACAA ACCACCACCTE TACAGAGGAC CACCAGACAACAACAACACACACCACCT TACACCAGGAC CACCACCAGA GACCCCCAGA CACCACCACCACCACCACCACCACCACCACCACCACCA		CCACCTCTAC	CAGGGCTGCC	AGGTGGTGCA	GGGAAACCTG	GAACTCACCT	ACCTGCCCAC	CAATGCCAGC	CTGTC	225
CTICTICAGG GATATCCAGG AGGTSCAGGG TAGGTSCTA ATGCTTACA ACCAAGTAGG GCAGTTCCA CTCA TPHELEUGIA ASPITEGIAGI GLVALGIGIGI YTYVALIEU ILEALARISA SIGINVALAY GGINVALFTO LEUGI GAGGCTGCGG ATTGTGCGAG GCACCAGCT CTTTGAGGAC AACTATGCCC TGGCCGTGT AGACAATGA GACCC AATGLEWAY ILEVALAYGG IYTHTGIGIGU UPHEGIUMSP ASTTYTALLE ENALAVALLE MASPASHGIY ASPIT CCTGAACAAT ACCACCCCTG TCACAGGGC CTCCCAGGA GACCTGCGGG AGCCCAGCT TGGCCAGCT TGAGCAGCT ACAGG GACCTGAACAAT ACCACCCCTG TAACAGGGC CTCCCAGGA GACCTGCGGG AGCCCAGCT TTTTGTGAGA GACAT CATCTTGAAA GGAGGGGTT TGATCCAGG GAACCCCCAG CTCTGCTACC AGGACCAGAT TTTTGTGAGA GACAT UTLELEULYS GIYGIYVALL EUILGINAY GASPFOOLD LEUCYSTYG INASPITHI eleUTTPLYS ASPIT CTCCACAAG AACAACCAGC TGCCTCCAC ACGATTCTAGA GACACCAGAT TTTGTGGAG GACAT UTLELEULYS GIYGIYVALL EUILGINAY GASPFOOLD LEUCYSTYG INASPITHI eleUTTPLYS ASPIT CTCCACCAGA AACAACCAGC TGCCTCACC ACGATTCTAGA GACACCAGAT TTTGTGGAG GACAT UTLEAGA ACAACCAGC TGCCTCCAC ACGATTCTAGA GACTCACCAGT CTCCCACCCTG CTCCCCAGC GCCCCCAGCGC CACTGCTCCACAGA GACTCCCACCC CTCGGGAGAA ACTARCACC ACGATTCTAGA GACTCCCAGC CTCCCAGCG CACTCCCCCC CCTCCAAGAGAAC ACGATTCTAGA GACTCCCACCC CTCCCAAGCAC ACGATTCTCACACACACACACACACACACACACACACACA		gHisLeuTyr	GlnGlyCysG	lnValValGl	nGlyAsnLeu	GluLeuThrT	vrLeuProTh	rAsnAlaSer	LeuSe	
REPRIENCIA ASPITECIAG CACCACCT CTTTGAGGAC AACTATGCCC RAGGCTOCGG ATTGTCCGAG CACCACCT CTTTGAGGAC AACTATGCCC RATGTCCGAGG CACCACCACT CTTTGAGGAC AACTATGCCC RATGTCCGAGGACCT TICACAGGGGC CTCCCCAGGA GCCCTGAGGACCT AGACAATGGA GACCA RACCACCCTG TCACAGGGGC CTCCCCAGGA GCCCTGCGGG ACCTCCCAGGA GCCCTGAGCACTA TACCACCCCTG TCACAGGGGCCCCAGGACCCT ACAGGA CLEUASNASH THATFIFPOV AITHTGIVAL SEPTEORILY GLYLEUAYGG LULEUGINEU WASPASHGIV ASPPT CATCACACAGA ACAACCACC TGACCTCCAC GACACCCCCG CTCTGCTACC AGGACACCAT TTTGTGGAGG GACAT CTTCCACAGA AACAACCAC TGACTCTCAC AGGATGAGACCAC CTCTGCGACGTC CACCCCCTGT TCCC CACCTACAAGA AACAACCAC TGACTCCCAC GACACCCCC CTCTGGGCCTG CACCCCCTGT TCCC CACCTGCAAGA GACAACCACC TGACTCCCAC GAGATTGAGA AACAACCAC TGACTCTCAC AGGATTAGAA AACAACCACC TGACTCTCAC AGGATTAGAA ACAACCACC GACTCCCAGG GCCCCCCCG GCTCCACGGAG GAGTTCTGAG GATTCTGAG GATTCTCAAG GCCTGACGGC GACTGCCTGC GCCTCAAGG GCCCTCTGACTGC CACCTGACTGC GCCTCAAGA GGCTCCCCCC GCTGACGGAG GACTCCCCCACCTGT TCACCACC AGTGGCCCCC ATTGACTGC GCCTCAAGG GCCCTCACCCAGG GCCCC CACTGACTGC GACCTCCCACC CACTGACTGC CACCTACCCAGA GAGCTCCCCCCCC CTCGACCCCC CTCGACCCCC CTCGACCCCCCCCC CTCGACCCCCCCC CTCGACCCCCCCCC CTCGACCCCCCCCCC		CTTCCTGCAG	GATATCCAGG	AGGTGCAGGG	CTACGTGCTC	ATCGCTCACA	ACCAAGTGAG	GCAGGTCCCA	CTGCA	300
GAGGTGCGG ATTOTGCGGG CCACCCAGT CTTTGAGGGA AACTATGCCC TGCCGTGCT AGACAATGGA GACCC AATGGAGGT TATCTGALE UPHGLUASP ASITYTALAL EUALAVALLE UASPASRGLY ASPT GCTGAACAAT ACCACCCCGT TCACAGGGG CTCCCAGGA GACCTCAGGA AACTGCAGGT TGAGCCCTC ACAGA CACCTAAACAA GAAGGGGTT TGAGCCAGG GACCCCCAG GCCTCCTGCAGA GACCTCAGGT TGAGCCCTC ACAGA GACTAACAACAACACGC TGCCTCCACAG GACCTCAGGT TATCTGAAGGCTT TATCTGAGGGGACT TGATCCACAGG GACCTCCCAGG GACCTCCCAGG GACCTCCCAGG GACCTCCCAGG GACCTCCCACAGA AACAACCAGC TGCCTCTCAC ACGATAGAC ACCAACCGCT CTCCGGGCCT CACCCCTT TCCC GACCTT TCCC ACTGTTGAGA GACAACACCAC TGCCTCTCAC ACGATAGACA ACCAACCGCT CCCGCTCAGC GCTCCAGGAG GACTTCTGAG GACTTTCAGA GACTATCAGA GACTACCACC GACCTCCCA CTCGGGAGA GAGTTCTGAG GACTTTCAGA GCCTGCCCC GCTCGGAGGA GAGTTCTGAG GACTTTCAGA GCCTGACCGC GCTCCAACACCAC GACTGCCTCC ACTGACCAC GACTGCCTCC CACTGACCAC GACTGCCTCC CACTGACCAC GACTGCCTCC GCTCCACCCC CTCCACCCC CACCTGCTCC GACTGCCTCC ACTGCCTCCA CTCCACCACC GACGGCCCCT TACAACCAC ACAGCACCTT TGAGTCCAC CACCACCACCT TGCCCCCACCCC TTCAACACAC ACAGCACCTT TGAGTCCAC GCCCAACCCC GACGGCCCCT TACAACCAC ACAGCACCTT TGAGTCCAC GCCCAACCCC TTCAACCAC ACACCACCACCT TTCAACCAC ACACCACCACT TTCAACCAC ACACCACCACT TTCAACCAC ACACCACCAC TTCAACCAC ACACCACCACT TTCAACCAC ACACCACCAC TTCAACCAC ACACCACCACCT TTCAACCAC TTCAACCAC ACACCACCAC TTCAACCAC TTCAACCAC ACACCACCAC TTCAACCAC ACACCTACAC TTCAACCAC ACACCTAC ACACCACAC ACACCACAC TTCAACCAC ACACCTAC ACACCACAC ACACCACAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACCAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACAC TTCAACCAC ACACCACAC		rPheLeuGln	AspIleGlnG	luValGlnGl	yTyrValLeu	IleAlaHisA	snGlnValAr	qGlnValPro	LeuGl	300
ARTICLEMARY ILEVALAGE LYTHROLIME UPHEGLUARY ASTROYALLE UNASPASHIJY ASPPT GCTGACCACCCCTG TCACAGGGGC CTCCCCAGA GCCCTCCCCGG AGCTCACCGG TCGACCACCCCTG TCACAGCCCT ACCAC CLEUARNASH THITHFORD ALTHROLIMAL SEPTPODY GLYLLUARYG LULEUGLILLE UNASPSETLEU THIGH GATCTTCAACAG GAGCGGGTCT TGATCCACG GAACCCCCAG CTCTCCTACC AGGACACGAT TTTTTGGAAG GACAT LITLELEULYS GLYGLIVALLE GULIGILARY ASARDOGIL LEUCYSTYG INASPTHITLE GATCTTCACAGA AACAACCAG TGCTCTCAC ACGATAGAC ACCAACCGCT CCACGCCCTG TCTCCCACAG GATCTCCTAC ACGATAGAC ACCAACCGCT CCACGCCCTG TCTCCCACAG GATCTCTCAC ACGATCACCAG CCCCCCCG GCTGGGAGA GATCTCCAGA ACCAACCAGC TGCCCCG GCTGGAGAGA GATCTCCAGAGACACCAG GCCCCCCCG GCTGGAGAGA GATCTCCAGACCAC ACTGCCCCG GCCCCCG GCCCGGAGAGA GATCTCCAGACCAC CACTGCCCC GCCCCCC GCCCGGAGAGA GATCTCCAGACCAC CACTGCCCC GCCCCCCC GCCCCGAGAGA GACCCCCCC CCCCCACCCCC CTCCACACCAC CACTGCCCC GCCCCCCACCCC CTCCACCACCCC CTCCACCACCCC CTCCACCACCCC CTCCACCACCCAC		GAGGCTGCGG	ATTGTGCGAG	GCACCCAGCT	CTTTGAGGAC	AACTATGCCC	TGGCCGTGCT	AGACAATGGA	GACCC	375
GCTGACCAT ACCACCCCTG TCACAGGGG CTCCCCAGA GCCTGCGG AGCTCAGCT TCAAACCTC ACAGA ACACCACCTT TATTCTAPA AGAGGGGTC TGATCCAGG GACCCCCAG CTCTGCTACC AGAGACACAT TTTGTGGAAG GACAT UTleleulys GlyGlyValL eulleGlnar gamprodin LeuCysTyrg InaspThr11 eLeuTrplys Asp11 CTCCCACAA AACAACCAGC TGCTCTCAC ACTGATAGAC ACCAACCCCT CTCGGGCCTG CCACCCCTTT TCCC AGACACCAGC TGCTGCTCAC ACTGATAGAC ACCAACCCCT CTCGGGCCTG CCACCCCTTT TCCC AGACTGGTAAG GACAACCAGC TGCTGCTCAC ACTGATAGAC ACCAACCCCT CTCGGGCCTG CCACCCCTTT TCCC AGACTGGTAAG GACTCCCCCT GCTGGGGAGA GAGTTCTTAGG GATTGTCAGA GCCTGACCG CACCTGTCTGT GCCG AMECYGLYS GlySeragC ysTpGlyGl userserdlu AspCysGlns erLeuThrar gThrValcys SerPr GATTGTAAG GCCTCCACCG GCCCCCCC CACTCACCG CACCTGTCCC CACCCACCCC CACCCACCCCC CACCCACCCC CACCCACCCC CACCCACCCCC CACCCACCCC CACCCACCCC CACCCACCCCC CACCCACCCCCC		nArgLeuArg	IleValArgG	lyThrGlnLe	uPheGluAsp	AsnTvrAlaL	euAlaValLe	uAspAsnGlv	AspPr	
OLEMASINASTI THITHIPTOV AITHIGIJVAI aSEPTROGLY GLYLEUATGG LULEUGINLE WATGSETLEU THITGG GACATT TOTTCACCAGG GACAGGGTTT TGATCCAGCG GACACCCCAG CTCTGCTACC AGGACAGAT TTTGTGGAAG GACAT UTLEULIANG GLYGLYVALL EULIGINAY GASHPOOLIN LEUCYSTYTG INASTITUTI ELEUTIPLYS ASDII CTCCACAAG AACAACCAG TGGCTCTCAC ACTGATAGAC ACCAACCGCT CTCGGGCCTG CCACCCCTGT TCTCC 600 ePheHislys AsnAsnGILL euAlaleuTh rleulieAsp ThrAshArgs erArgalacy stisprocys SerPr GATGGTTAGA GCCTCCACCCG CTCGGGGAGA GAGTTCTGAG GATTGTCAGA GCCTGACCGC CACTGCTGT GCCG 675 MECCYSLYS GLYSERAFG YSTTGIYGI USERSERGIU ASPCYSGINS ERLEUTHTAY GTMVAICYS Alaci TGGCTGTGCC CCCTCCACAG GCCCACCTCCCC CACTGACTGC CACTGACTGC CACTGACTGC CCCTGCACG GCCC YGLYSHISSER ASPCYSLYS GLYPTOLEUP OTTMASPCYS CYSHISGIUG INCYSALAAI AGLYCYSTTH GLYPT CAACCACACCGC CCTCCCACA CTCCACCACA CTTCCACCACA CTGCACCACC CTGCT ACCACCACA CACGCACCTC GCACCACCC CTGCT 825 CLYSHISSER ASPCYSLEUA IACYSLEUHI SPHEASHHIS SERGIYIIC YSGULLEUHI SCYSPTOAIA LEUVA CACCTACAAC ACAGCACACA CTTGACCACA CTTCCACCCC GAGGGCCCGT ATACACTTCG CCCACCTGT GTGAC 900 LTHITYASH THRASPTHYP HEGIUSETME TPTOASHTOR GAGGGCCCGT ATACACTTCG CCCAACCAA GAGGT TTAACACCA CTTCCACCCC TACACCACA CACGCACGAG CATTGCACCAC CTTCCTCCCC TACACCACA CACGCACGAG CACCACCACC TTTTTAACGGA CGTGGGATC TCCACCCTCT CTCCCCCCT GCAACACCA GAGGT TAACACTACC TTTTTAACGGA CGTGGGATC TCCACCCTCT CTCACCCCCT GCAACACCA GAGGT TAACACTAC TTTTTAACGGA CGTGGGATC TCCACCCTCT CCCCCCCT GCACACCAA GAGGT TAACACTACC TTTTTAACGGA CGTGGGATC CACCACCACCA CTCCTCCCCCT GCACAACCAA GAGGT TAACACTACC TTTTTAACGGA CACCACCAACA AACCCCTGTG CCCAACCACAC AACCCACCACAC TTTTCTCCCCG GAACACCAA CACCGTTGT CACACCACAC		GCTGAACAAT	ACCACCCCTG	TCACAGGGGC	CTCCCCAGGA	GCCTGCGG	AGCTGCAGCT	TCGAAGCCTC	ACAGA	450
GATCTTGAAA GAGGGGTCT TGATCCAGCG GACCCCCAG CTCTCCTACC AGGACAGGAT TTTTTGGAAG GACAT UTILE GLIPS ASPIL CTTCCACAAG AACAACCAC TGGCTCTCAC ACTGATAGAC ACCAACCCCT CTCGGCCTG CACCCCTGT TCTCC GPHEHISLYS ASMASRGIAL euAlaleuTh rieuTleAsp Thrashargs erargalacy shisprocys Serpr GATGTGTAGA GCCCCCCC GCACCGTGT GCCCG GACCTGTGTGC GCGGGAGA GAGTTCTGAG GCTCGCCGC GCCCGCGGAGA GAGTTCTGAG GCCTCACCGC CACTGCTGT GCCCG GMECCYSLYS GlySerArgC ysTrpGlyGl userSerGlu AspCysGlns erLeuThrar gThrValcys Alagi TGGCTGTGCC GCGCGCAGG GCCCC CACTGACGG GCCCC CACTGACGG GCCCC CACTGACGG GCCCC CACTGACGG GCCCC CACTGACGG GCCCC CACTGACGG GCCCC CTGCAGGA AACACCACT TGAACCAC ACTGCCAGG CACTGCACGG GCCC CACTGACGG GCCCC CTGCAGGA AACACCACT TGAACCAC ACTGCACGG GCCCCAGCC CTTCAACCAC ACTGCACACC AACGCACCT TGCACCAC CTCCAACCAC ACTGCACACC AACGCACCT TGAACCAC ACCACACACA AACGCACTT TTCAACCAC ACCACACACA AACGCACTT TTCAACCAC CTTCAACCAC ACCACACACA AACGCACTT TTCAACCAC CTTCAACAC ACCACACACA AACGCACTT TTCAACCAC ACCACACACA AACGCACTT TTCAACCAC CTTCAACAC ACCACACACA AACGCACTT TTCAACCAC CTTCAACAC ACCACACCAC		oLeuAsnAsn	ThrThrProV	alThrGlyAl	aSerProGly	GlyLeuArgG	luLeuGlnLe	uArgSerLeu	ThrGl	
UILELeulys GlyClyVall euIleGinAr gAsnFroGin LeuCysTyrG inAspThrII eleuTrplys AspII CTTCCACAAG AACAACCAGC TGGCTCTCAC ACTGATAGAC ACCAACCGCT CTCGGGCCTG CCACCCCTG TCTCC ePheHislys AsnAsnGinL euAlaleuTh rieuIleAsp ThrAsnArgS erArgAlady sHisProCys SerPr GATGGTHAG GGCTCCCCT GCTGGGGAG GAGTTCTGAG GATTGTCAGA GCCTGACCGG CACTGTCTGT CCCG GMetCyslys GlySerArgC ySTrpGlyGl uSerSerGlu AspCysGins erLeuThrAr gThrValcys AlaGi TGGCTGTGCC CGCTGCAAGG GGCCACTGCC CACTGACTGC TGCCATGAGC AGGTGCTCC CGCCTGCACG GGCCC yGlyCysAla ArgCyslysG lyProLeuPr oThrAspCys CysHisGluG inCysAlaAl aGlyCysThr GlyPr CAACCACTCT GACTGCCTG CCTCCTCCA CTTCAACCAC AGTGGCACTC GTGAGCTGCA CTGCCCAGCC CTGGT OLySHisSer AspCysLeuA laCysLeuHi SPheAshHis SerGlyIlcc ysGluLeUHi ScySProAla LeuVa CACCTACAAC ACAGACACT TTGAGTCCAT GCCAATCCC GAGGGCCGT ATACATTCG CGCCTGCAAC CTGGT CCCTGTCC TACAACTACC TTTCAAGGA GGTGGGATCT TCCACCCAC TCCACCACACA GAGGT TTTAAGTTCAT GCCGATCCC TCCACCACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CACCACCAA GAGGT TATACATTCG CTCACACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACCA GAGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATTCG CTCACCACCA GGGGT TATACATCAG CTCCACCACA GGGGT TATACATCAG CTCCACCACA GGGGT TATACATCAG CTCCACCACA GGGGT TATACATCAG CTCCACCACA CTCCCCCACCACACC TTTCGCTG CAGACACAC AGAGGTTTCA TGCGGCCCAAT ATCCACGAGT TTTCGCTGCT CAAGCACCACA CTCCCCCGC CAAGCACACC TTTCGCTGCACACAC CTCCCCACCACACC TTTCGCTGCACACCA CTCCCCCCT CAAGCACCACC TTTCGCACATC CAGGCACCACACC TTTCGCCACACACC TTTCGCCCACCACACC TTTCGCCCCCC CACCACACC TTTCCACCACCA CACCTCCCCCC TCACCACCACC TCACCACCACC TTTCACCCCCCC CACCACCACC TTTCCACCACC TTTCCACCACC TTTCCACCACC TTTCACCCCCCC TCA		GATCTTGAAA	GGAGGGGTCT	TGATCCAGCG	GAACCCCCAG	CTCTGCTACC	AGGACACGAT	TITGTGGAAG	GACAT	525
CTICCACAA ACAACCAC TGGCTCTAC ACTGATRACA ACCAACCGCT CTGGGCCTG CCACCCCTGT TCTCC ePhHislys AsnAsnGnL ewAlaleuth rleulleAsp thrashargs erargalacy sHisprocys SerPr GATGGGTAGG GGCCCCCGC GCTGGGGAGA GAGTTCTGAG GATTGTCAGA GCCTGACGG CACTGTCTGT GCCG OMECCYSLys GlySerArgc ysTrpGlyGl uSerSerGlu AspCysGlns erleuthrar gThrValCys AlaGI TGGCTGTGC CGCTGCAAGG GGCCACTGCC CACTGACTGC TGCCATGAGC AGTGTCTCC GGCCC CAGGCACTGC GCCCC CTGAAGAAA ATGCYSLysG lyProleupr ThrAspCycglus lnCysAlaAl alglySyThr Glypr CAAGCACTCT GACTGCCTG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGC CTGGT ASpCysLeuA laCysLeuHi sPheAsnHis SerGlyIleC ysGluLeuHi SCysProAla LeuVa CACCTACAAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CGCCACCTGT GTGAC LThTTYTASn ThrAspThrP heGluSerMe tproAsnPro GluGlyArgT yThrPhedl yAlasecys ValTh TGCCTGTCCC TACAACACA CACGACACAC TTTCTACGGA GGTGGGATCC TGCACCCTG TCTGCCCCT GCACAACCAA GAGGT LThTALaGlu AspGlyThrG lnArgCysGl uLysCysSer LysProCysA laArgValCy sTyrGlyLeu GlyMe CGACACCAGAG GATGGAACA AGCGGTTGA GAAGTCCAG AGAGCCCTTG CCCAGATGTG CTATGGTCG GCAC CAGGCAGAG GATGGAACA AGCGGTTGA TGCGGACCA AGCCCTTG GCCAATCTG GCCAAT TTTGG CGAGCCTGCA TTTCTCCCG AGAGCTTTA TGGGGACCA ACCCCAAT ATCCAGGAGT TTCCTGCCC CAGCAGAGAC TTTCTCCCG AGAGCTTTA TGGGGACCA ACCCCCAAT ATCCAGGAGT TTCCTGCCC CAGCAGAGAC TTTCTCCCG AGAGCTTTA TGGGGACCA ACCCTCCAACA CTCCCCCCT CAGCCAGG CACC YSERLEUAla PheLeuProG luSerPheab gGlyAspPro AlaserAnt halaProle uGlnProGlu GlnLe CCAAGTGTTT GAGACTCTG AAGAGATCA CAGGTTCAAC AGGTTACCTG GCCCATCTC GCCCCCCT CAGCCAGG CACCT YSERLEUAla PheLeuProG luSerPheab gGlyAspPro AlaserAnt halaProle uGlnProGlu GlnLe CCAAGTGTTT GAGACCTCG AAGAGACAC AGGCTTCAC CAGCCCCCC CAGCCAGG CACCT YSERLEUAla PheLeuProG luSerPheab gGlyAspPro AlaserAnt ProTagccca CAGCCTCCCC CAGCCAGG CACCT YSERLEUAla PheLeuProG luSerPheab gGlyAspTle LeuHislashang lyAlatyrse rLeutProLeu GlnCal CCAGGCCTC CAGAGACCAC CGGGCCCC CACCAACC TCCCCCCC CAGCACCAC CGCCCCCCC CACCACCAC CCCCCCCC		ulleLeuLys	GlyGlyValL	euIleGlnAr	gAsnProGln	LeuCysTyrG	lnAspThrIl	eLeuTrpLvs	AspIl	
ePheHislys AsnasnGlnL euAlaleuTh rLeuIleAsp ThrAsnargs erArgAlacy sHisProcys SerPr GATGITARAG GGCTCCCGCT GCTGGGGAG GATTICTGAG GATTICTCAGA GCCTGACCG CACTGICTIT GCCG GMECCYSLys GlySerArgC ysTrpGlyGl uSerSerGlu AspCysGlns erLeuThrAr gThrValcys AlaGI TGGCTGCCC CGCTGCAAGG GGCCACTGCC CACTGACTC TGCCATGAGC AGTGTGCTG CGGCTGACGG GGCCC YGlyCysAla ArgCyslysG JyProLeuPr oThrAspCys CysHisGlug InCysAlaAl aGlyCysThr GlyPr CAAGCACTCT GACGCCTGG CCTGCCTCCA CTTCTAACCAC AGTGGCATCT TGCAGCCTCA CTGCCAGCC CTGGT 825 OLYSHisSer AspCysLeuA laCysLeuHi SPheAsnHis SerGlyIleC ysGluLeuHi SCysProAla LeuVa CACCTACAAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGT ATACATTCGG CGCCAGCTGT GTGAC CACCTACAAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CGCCAACCAA GAGGT THTATYASN ThrAspThrP heGluserMe tProAsnPro GluGlyArgT yrThrPheGl yAlaSerCys ValTh TGCCTGTCC TACAACTAC TTTCTACGGA CGTGGGATCC TCCACCCCTG TCTACCCCCT GCACAACCAA GAGGT TALACYSPTO TyrAsnTyrL euSerThrAs PVALGIySer CysThrLeuV alcysProLe uHisAsnGln GluVa GAACGACAGG GATGGAACA AGCGGTGTGA GAAGTGCAGA AAGCCCTGT CCCCAATCTG CTATGGTCTG GCAT 1ThrAlaGlu AspGlyThrG inArgCysGl uLysCysSer LysProCysA laArgValCy styrGlyLeu GlyMe GGAGCCTGGCA TTTCTGCCGG AGAGCTTTCA CAGTGCCAAT ATCCAGGAGT TTCCTGCCTG CAAGAAGAAC TTTCTGCCGG AGAGCTTTCA TGGGGACCCA GCCTCCAACCAA GAGCT TTCTTCTGCCGG AGAGCATTTCA TGGGGACCCA GCCTCCCACC CAGCCAGGG CAGCT YSERLeuAla PheLeuProG luSerPheAs pGlyAspPro AlaSerAsnT hrAlaProLe uGlnProGlu GlnLe CCAAGTGTT GAGACTCTG AAGAGAATCA AGGGACACA CTGCCCCCCT CCACCAGGG CAGCT YSERLeuAla PheLeuProG RAGACATCC AGGGACACA CTGCCCCCCCCCCC CACCCACGAG CAGCT USERVAIPHe GluThrLeuG loGluIleTh rGlyTyrLeu TyrILeSerA laTpProAs pSerLeuPro AspLe CACCCTCTC CAGAACCAC CGCTCCCAC CACCACGC CACCACAC CTGCCCCCC CACCACAC CTGCCCCCC CACCACAC CTGCCCCCC CACCACAC CTGCCCCCACCCCC CACCACAC CTGCCCCCCCCCC		CTTCCACAAG	AACAACCAGC	TGGCTCTCAC	ACTGATAGAC	ACCAACCGCT	CTCGGGCCTG	CCACCCCTGT	TCTCC	600
GATGOTAAG GCCCCCCCT GCTGGGGAAA GAGTTCTGAA GATTGTCAGA GCCTGACCGC CACTGTCTGT GCCG OMETCYSLyS GlySerArgC ySTTpGlyGl USERSGUA BACYSGUAS ETLEUTHTAT gThrValCyS AlaGI TGGCTGTGCCC CGCTGCAAGG GCCCACTGCC CACTGACTGC TGCCATGAGC AGTTGGCTGC CGGCTGCAGG GCCC yGlyCySAla ArgCysLysG lyProLeuPr oThrAspCys CysHisGluG lnCySAlaAl aGlyCysThr GlyPr CAAGCACTCT GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC CTGGT CACCTACAAC ACACACACGT TTGAGTCCAT GCCCAACCC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC CTGGT CACCTACAAAC ACACACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CCCCACCTGT GTGAC CACCTACAAAC ACACACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CCCCACCTGT GTGAC CTALACTSAC TTTCTACGGA CGTGGGGATC TCCACCCTG TCTGCCCCCT GCACACCAA GAGGT TALACTSAC TTTCTACGGA CGTGGGGATC TCCACCCTG TCTGCCCCCT GCACACCAA GAGGT CALACCACAC CTTCTACACAC AGGGTGTGA CACACCACG CTCTCCCCCT GCACACCAA GAGGT TALACTSAC TTTCTACGGA CGTGGGGATC TCCACCCTG CTCTCCCCCT GCACACCAA GAGGT TALACTSAC TTTCTACGGA CGTGGGGATC TCCACCCTG CTCACCACTG CTCACCACAC GACACACAACAC CTCCCCCGT CTACGGTGG GCAT TALACTSAC AGCGGTGTA CGGCAGTTAC CAGTGCCAAT ATCCACGAGT TTCCTGCCTG CACAACAAACATC TTTGG GGACCACTGC CAGAGAGTAC CGGCAGTTAC CAGTGCCAAT ATCCACGAGT TTCCTGCCTG CAACAAACATC TTTTGG TLTTALACAC TTTCTCCCGG AGAGCTTAA TCCACGACAC CTCCCCCGCT CACCCACGA CACCTCCACACA CTCCCCCGCT CACCACACA CTCCCCCGCT CACCACACA CTCCCCCGCT CACCACACA CTCCCCCGCT CACCACACA CTCCCCCGCT CACCACACA CTCCCCCGCT CACCACACA CACCTTTTTGG CACAACACACACA CTCCCCCGCT CACCACACA CACCTCTC CACCACACA CACCTTCC CACCACACA CACCTCTC CACCACACA CACCTCTC CACCACACACA		ePheHisLys	AsnAsnGlnL	euAlaLeuTh	rLeuIleAsp	ThrAsnArdS	erArgAlaCv	sHisProCvs	SerPr	-
OMECVSLYS GİYSETARGC YSTTPĞİYGİ USERSERĞİU ASPCYSĞİNS ELEUTÜTRA ƏTRIVALÇYS ALAĞI TÜĞCÜTÜĞÜYÜYSALA ARÇOYSLYSĞ İYPTOLEUPT ÖTÜTARÇOYS CYSHİSĞİÜĞ İNCYSALAAL AĞIYÇYŞTR ĞİYPT CAAĞCACTCT ĞACTĞCCTĞ CCTĞCCTĞA CTTÇACÇAC AĞTĞĞCATCT ÜÇÇAĞLAL AĞIYÇYŞTR ĞİYPT CAAĞCACTCT ĞACTĞCCTĞ CCTĞCCTĞA CTTÇACÇAC AĞTĞĞÇATCT ÜTĞAÇCTĞC CTĞĞCÇĞĞÇ CTĞĞT AŞÇOYSLYSİ İYPTOLEUPT ÖTÜTARÇOYS ÇİŞHİSĞİÜĞ İNCYSALAAL AĞIYÇYŞTR ĞİYPT CAAĞCACTCT ĞACTĞCÇT ÇÜÇÇÜĞ CCTĞÇÜÇÜĞ CTĞĞÇACÇÜĞ TÜĞAÇÇATÇC ÜTĞAÇÇAÇÇÜ TÜĞAÇÇATÇÜ ÇÜÇĞÇAĞÇTĞ TÜĞAÇ CAÇCTAÇAAC AŞÇOYSLEUH İSÇYSLEUHİ SPREASHHİS SERĞİYİLEÇ YSĞÜLLEUHİ SÇYSPOALA LEUVA CAÇCTAÇACA ÇAÇĞAÇÇÜĞ TİÇAĞÇAÇĞÜ TÜĞAÇÇATÇÇÜ ÇÜÇĞAÇÇÜĞ TÜĞAÇÇAÇÇÜ TÜĞAÇÇAÇÇÜ ÇÜÇĞAÇÇÜĞ TÜĞAÇÇAÇÇÜ ÇÜÇĞAÇÇÜĞ TÜĞAÇÇATÇÜ ÇÜÇĞAÇÇÜĞ ÇÜÇÇAĞÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞAÇÇÜĞ ÇÜÇĞÇĞÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇĞÇÜĞ ÇÜÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜÇ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜĞ ÇÜÇÜ ÇÜÇÜ ÇÜÇÜÇÜĞ ÇÜÇÜÇÜĞ ÇÜÇÜ ÇÜÇÜ ÇÜÇÜÇÜĞ ÇÜÇÜÇÜ Ç		GATGTGTAAG	GCTCCCCCT	GCTGGGGAGA	GAGTTCTGAG	GATTGTCAGA	GCCTGACGCG	CACTGTCTGT	GCCGG	675
TGCATGACE GGCTGCAAGG GGCCC GGLYCYALA ARGYSLYSE JYPTOLEUPT GTHASPCYS CysHisGluG IncysAlaAl AGIYCYSTHR GIYPT CAAGCACTC GACTGCCTCC CTCCCTCCA CTTCAACCAC AGTGGCACTC GTGAGCTCCA GTCAGCTCC CTGGT GLYSHISSER ASPCYSLEUA LGCYSLEUHI SPHEASHHIS SERGLYILC GTGAGCTCCA GTCAGCTGC GTGAGCACGT TTGAGTCCAT GCCCAATCCC GAGGGCGGT TAACATTCG GCCAGCTGT GTGAC GCCAGCTGT GTGAC GCCAGCTGT GTGAC GCCAGCTGT GTGAC GCCAGCTGT GTGAC GCCAGCTGT GTGACCCCC TTTCTACCGC TTTCTACCGA GAGGGTCC GACACACAA GAGGT TTCTCACCCC GCCAGCTGTG GCCAACACAA GAGGTTCAC GCCAGCTGTG GAACCACAAG GAGGGTCA GGCAGGTTGA GAGGGTCAC GGCAGGTTGA GAGGCCTTGA GAGGGTCAC GGCAGGTTAC GGCAGGTTAC GGCAGGTTAC GGCAGCTGTC GAGACCTGCA GAGCCTTGAC GAGGCCTGCA GAGCCTTGA TTCTGCCCCC GAGACACACA TTCTCGCCCC GACAACACA GGCACCACTGC GCCAGCTGT GCCACCCT GCCACCTCC GCACACACACA GCCCACCTG GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCACCTGC GCCACCTGCC GCCACCTGCC GCCACCTGCC GCCCCCCCCC CACACACACA TTCTCGCCCC CACACACACA TTCTCGCCCC CACACACACA TTCTCGCCCCC CACACACACA TTCTCGCCCC CACACACACA TTCTCGCCCC CACACACACA TTCTCGCCCC CACACACACA TTCTCGCCCC CACACCACAC		oMetCysLys	GlySerArqC	ysTrpGlyGl	uSerSerGlu	AspCvsGlnS	erLeuThrAr	oThrValCvs	AlaGl	
YGİYCYSAİLA ARGYSİLYSĞ İYPFOLEUPT OTHRASPCYS CYSHİSGİLG İNCYSAİLALİ AĞİYCYSTHE ĞİYPF CAAĞCACTCI GACTGCCTĞĞ CTĞCCTĞCA CTTCCAĞCC CTĞĞT ÖLÜŞKİİSSER AŞPCYSLEUA İACYSLEUHİ SPHEASHİİS SERĞİYİLEC YSĞİULEUHİ SCYSPFOALA LEUVA ÇACTACACA CACÇACAÇAC TÜĞAĞCTĞĞA TARCATTĞĞ CĞCCAACTĞT ĞIĞAC ÇACÇAÇACTĞI ĞIĞAC İİTİTİYRASI THRASPTHEP HEĞİUSERME EPROASINFO ĞÜLĞİYARĞI TÜRÜĞAÇÇAĞÇA TARCATTĞĞĞ CĞCCAACCAĞ ĞAĞĞĞ ÇÜLÜĞÜLÜNÜN TÜRÜĞÜNÜN TÜRÜNÜN TÜRÜĞÜNÜN TÜRÜNÜN T		TEGETETECC	CGCTGCAAGG	GGCCACTGCC	CACTGACTGC	TGCCATGAGC	AGTGTGCTGC	CGGCTGCACG	GGCCC	750
CAAGCACTCT GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC CTGGT GLOLYSHISSER ASPCYSLEUA LACYSLEUHI SPHEASNHIS SERGLYLEUY YSGLULUHI SCYSPTOALA LEUVA CACCTACAAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CGCCAGCTGT GTGAC GCCCAATCCC TTGAGTCCAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CGCCAGCTGT GTGAC GAGGACCAC TTGAGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC TCGACCCTG TCTGCCCCCT GCACAACCAA GAGGT ATACATCACG GATGGAACCA GAGGT TTCTACGGA CGTGGATCC TCGACCCTG TCTGCCCCCT GCACAACCAA GAGGT GACACACAGA GATGGAACAC AGCGGTGTGA GAAGTGCACC AAGCCCTTGT CCCCAAGTTG CTATGGTCTG GGCAT TTACAACTACC GAGGACCACA AGCGCTGTGA GAAGTGCACC AAGCCCTTGT CCCCAAGTTG CTATGGTCTG GGCAT TTACAACTACAC AGCGCTGTAC CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACGTCA CAGGACCTA AGCCCTGTG CCCCAAGTTG CTATGGTCTG GGCAT TTCTGGCCGCA TTTCTGCCCGG AGACACACA TACCAAGGATT TCCTGGCTG CAAGAAGATC TTGG TCGCCACAT ATCCACGCAGT TTCCTGCCTG CAAGAAAGATC TTGG TCGCACCACA TTCTTGCGCACA AGACCTTGA TGGGGACCCA GCCTCCAACA CTGCCCCCCT CCACCACAGA CAGCTTGCA AGACCTTGA AAGACATCAC AGGTTACCTA TACATCCACACA CTGCCCCCCT CCACCACAGA CAGCTTCCCACACA CTGCCCCCACA CAGCCTGCCT GACCCT GACCCT GACCCTT GAGACCTTG CAGAACCTCC AAGACAATCAC AGGTTACCTA TACATCCACACA CTGCCCCCCT CAGCACCTGC GAACCT USCACCATTG GAGACCTCC AAGACAATCAC AGGTTACCTA TACATCTCAG CATGCCCCGA CAGCCTGCCT GAACCT USCACCATTG GAGACCTCC CAGGACCTAC CAGCGTCTTG CAGCACATTG CAGCACATTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CAGCACTTG CACCACATTG CAGCACTTG CACCACATTG C		yGlyCysAla	ArgCysLysG	lyProLeuPr	oThrAspCvs	CysHisGluG	lnCvsAlaAl	aGlvCvsThr	GlvPr	
OLYSHISSER ASPCYSLEUA laCysLeuHi SPheAsnHis SerGlyIleC ysGluLeuHi SCYSProAla LeuVa CACCTACAC ACAGACACGT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG GCCAGCTGT GTGAC 900 1ThTYTASN ThrAspThrP heGluSerMe tProAsnPro GluGlyArgT yThTPheGl yAlaserCys ValTh TGCCTGTCCC TACAACTACC TTTGTAGGGA GGGGGCCGGT ATACACTTCGG GCCAACCAA GAGGT rAlaCysPro TyrAsnTyrL euSerThrAs pValGlySer CysThrLeuV alCysProLe uHisAsnGln GluVa GACAGCAGAG GATGGAACA AGCGTGTGA GAAGTGCAGC AGCCCTGT CCCAGGTGTG CTAGGTCTG GGCAT 1050 1ThTALaGlu AspGlyThrG InArgCysGl uLysCysSer LysProCysA laArgValCy StysLysIle GAGCCTGGC GAGAGGGGA GGGCAGTTAC CAGTGCCAAT ATCCAGGAGT TTGCTGCGCTG CAAGAAGATC TTTGG 1125—tGluHisLeu ArgGluValA rgAlaValTh rSerAlaAsn IleGlnGluP heAlaGlyCy sLysLysIle PheGl GAGCCTGGCA TTTCTGCCGG AGAGCTTGA GAGGCCCAAT ATCCAGGAGT TTGCTGCCCTG CAAGAAGATC TTTGG 1125—tGluHisLeu ArgGluValA rgAlaValTh rSerAlaAsn IleGlnGluP heAlaGlyCy sLysLysIle PheGl GAGCCTGGCA TTTCTGCCGG AGAGCATTGA CAGGACCCA GCCTCCAACA CTGCCCCGCT CAGCCAGGG CAGCT YSerLeuAla PheLeuProG luSerPheAs pGlyAspPro AlaserAsnT hrAlaProLe uGlnProGlu GlnLe CCAAGTGTTT GAGACCTGG AAGAGATCAC AGGTTACCAA AGGTACCTA TACATCTCAG CATGGCCGGA CAGCCTGCCT GACCT USINVAILPHE GIUTHLEUG luGIUIIETh rGlyTyrLeu TyrIleSerA laTrpProAs pSerLeuPro AspLe CAGCGTCTTC CAGACACTG GAGCACACAC AGGTACCAC AGGTACACC AGCTCTCTC GAGACCCTG CAAGA ACAC TTGCACCACA AGCTGCCCTG CAAGAACCTG CAAGAACCTG CAGCGCCTG CACCACAC CTGCCCCGC CACCACCAC CACCTGCCT GACCT GACCT USINVAILPHA GUITHLEUG LIQUAIIETh rGlyTyrLeu TyrIleSerA laTrpProAs pSerLeuPro AspLe CAGCGTCTTC CAGAACCTG GACTGCCCTG GAGCACACAC CTGCACCACAC CTGCCCCAC AACAC TTGCGCCCAC AACAC TTGCGCCCC AACTGCCCTG CAAGAC TTGCGCCCC AACTGCCCTG CAAGAC TTGCGCCCC ACCTGCCC TTCCGCCAC ACCCTGCCC TTCCGCCAC ACCCTGCCC TTCCGCCAC ACCCTGCCC TTCCGCCCC ACCCTGCCC TTCCGCCC ACCCTGCC TTCCGCCCC ACCCTGCC TTCCGCCCC ACCCTGCC TTCCGCCCC ACCCTGCC TTCCGCCCC ACCCTGCCC ACCCTGCCC TTCCGCCCC ACCCTGCCC TTCCGCCCC ACCCTGCCC TTCCGCC ACCCTGCC TTCCGCCCCAC ACCCTGCCC TTCCCCCCACCAC TTCCCCCCAC ACCCTGCCCC ACCCTGCCC TTCCCCCCACCAC TTCCCCCCAC ACCCTGCCCC ACCCTGCCCC ACCCTGCCCC		CAAGCACTCT	GACTGCCTGG	CCTGCCTCCA	CTTCAACCAC	AGTGGCATCT	GTGAGCTGCA	CTGCCCAGCC	CIGGI	825
CACCTACACA ACAGACACT TTGAGTCCAT GCCCAATCCC GAGGGCCGGT ATACATTCGG CGCCAGCTGT GTGAC IThrTyrAsn ThrAspThrP heGluSerMe tProAsnPro GluGlyArgT yrThrPheGl yAlaserCys ValTh TGCCTGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC TGCACCCTCG TCTGCCCCCT GCACAACCAA GAGGT AlacysPro TyrAsnTyrL euSerThrAs pValGlySer CysThrLeuV alCysProLe uHisAsnGln GluVa GACAGCAGG GATGGAACAC AGCGGTGTGA GAAGTGCACA AAGCCCTGTG CCCGAGTGTG CTATGGTCTG GGCAT IThrAlaGlu AspGlyThrG inArgCysGl uLysCysSer LysProCysA laArgValCy sTyrGlyLeu GlyMe GGAGCACTTG- CGAGAGGTGA GGCCAGTTAC CAGTCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAACATC TTTGG GGAGCACTTG- CGAGAGGTGA GGCCAGTTAC CAGTCCAATA ATCCAGGAGT TTGCTGGCTG CAAGAACATC TTTGG GAGCCTGGCA TTTCTCCCCG AGAGCTTTGA TGGGGACCCA GCCTCCAACA CTGCCCCCCT CCAGCCAGAG CAGCT ySerLeuAla PheLeuProG luSerPheAs pGlyAspPro AlaSerAsnT hrAlaProLe uGlnProGlu GlnLe CCAAGTGTTT GAGACCTCG AAGAGATCAC AGGTTACCTA TACATCTCAG CATGCCCGGA CAGCCTGCCCTG GACCT uGlnValPhe GluThrLeuG luGluIleTh rGlyTyrLeu TyrIleSerA laTtpProAs pSerLeuPro AspLe CAGCGTCTTC CAGAACCTCC AAGAACATC GGGACGAATT CTGCACCAATA GGCCTTACTC GCTGACCCTG CAAGC USerValPhe GlnAsnLeuG lnValIleAr gGlyArgIle LeuHisAsnG lyAlatyrSe rLeuThrLeu GlnGl GCTGGCATC AGCTGCCTG GCCTGCCTC ACTGAGGGAA CTGGCCCTG CATCCACCAT AACAC yLeuGlyIle SerTtpLeuG lyLeuArgSe rLeuArgGlu LeuGlySerG lyLeuAlaLe uIleHisHis AsnTh CCACCTCTGC TTCGGCCAC CGGGCCCTG GGCCCAGCCC TTTCGGAACC CGCACCAACC TCTGCCCCAC ACTGC THISLEUCYS PheValHist hvValProTr pAspGllucu PheArgasnP roHisGlnAl aLeuLeuHis ThrAl TCCACCGCCA GAGGACGAGT GTGTGGCGGA GGCCTGGCC TCCCACCACC TGTGCCCCGC AGGGCACTCC TGGG AASnArgPro GluAspGluc ysValGlyGl uGlyLeuAla CysHisGlnL euCysAlaAr gGlyHisCys TtpGl TCCAGGGCCC ACCCAGGAT ATGTGAATCG CAGCTTCCTTC CGGGCCAGG AGTCCCTGGG TCAGCCCAGA GGAATCCCGA GGAATCCCGA GTACT THTCGAGCCCC CCAGGGGAT ATGTGAATCC CAGCCAGCT TTGCCGTGCC ACCCAGCAG GGAATCCCGA GGAATCCCGA GGACTGC TCGGGCCGC ACCCAGGAC TTTGGGCGGA GGACTGCT TGGGGCAG GGACTGCT TGGGGCAG GGACTGCC TGGGCCGCG AGGGCACTGC TGGGGCAGGAGT TTGGGGCGA GGCCTGCC TCCACCCAGC GGGCCCTGC TGGGCCAGGAGT TTGGGCGGA TTGGGCGGA GGCCTGCC TCCACCCAGC GGACC		oLysHisSer	AspCysLeuA	laCysLeuHi	sPheAsnHis	SerGlvIleC	vsGluLeuHi	sCvsProAla	LeuVa	-
Thrasphip hegluserme tproasnpro gluglyargt yrthryhegl yalasercys Valth TGCCTGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC TGCACCCTG TCTGCCCCCT GCACAACCAA GAGGT ralacyspro Tyrasntyrl euserthras pvalglyser cysthrleuv alcysprole uthisasngin gluva GACAGCAGAG GATGGACAC AGCGTGTGA GAAGTGCAGC AAGCCCTGTG CCCGAGTGTG CTATGGTCTG GGCAT 1050 lthralaglu aspglythr inargcysgl utyscysser tysprocysa laargvalcy styrglyleu glyme-GAGAGCACTTG CGAGAGGTGA GGGCATTAC CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAAGATC TTTGG 1125—CGIUHisleu Arggluvala rgalavalth rseralaasn lleginglup healaglycy stystysile PheGI GAGCCTGCA TTTCTGCCGA AGAGCTTGA TGGGGACCCA CCTCCAACA TGCCCCGCT CCAGCCAGAG CAGCT 1200 yserleula pheleuprog luserpheas pglyasppro Alaserant hralaprole uginproglu glinle CCAAGTGTTT GAGACCTGG AAGAGATCA AGGTTACTA TACATCTAG CATGCCCGGA CAGCCTGCCT GACCT 1275 uginvalphe Gluthrleug lugluileth rglytyrleu tyrilesera latripproas pserleupro Asple CAGCGTCTTC CAGCACCCG AAGAGAATCA CAGGTACCATA TACACAATG CGCCCGAG CAGCCTGCCT GACCT 1275 usinvalphe Gluthrleug lugluileth rglytyrleu tyrilesera latripproas pserleupro Asple CAGCGTCTTC CAGAACCTG AAGTAATCCG GGGAGAATT CTGCACAATG GCCCTACTC GCTGACCCTG CAAGG 1350 uservalphe Gluthrleug lyleuargse rleuargglu Leuglyserg GactGCCCT CATCCACCAT AACAC 1425 yleuglyile Sertripleug lyleuargse rleuargglu Leuglyserg lyleualale uilehishis Asinth CCACCTCTGC TTCGTGCACA CGGTGCCCT GACCAGCT TTCGGAACC CGCACCAAGC TCTGCTCCAC ACTGC 1500 rhiscleucys pheValhist hrValprotr paspGlnleu PheArgasnp rohisclinal aleuleuthis Thral TCAACCGCCCA ACCGAGT TGTGGCCCG GGCCCTGCC TGCCACCAGC TGTGCCCCG ACCCAAGC TTTGCTCCAC ACTGC 1575 aasintgpro GluaspGluc ysValglyGluglyLeuala Cyshisclinal eucysalaar gGlyHiscys Tttgcl TCCAGGGCCC ACCGAGGT TCAACCGCCCA ACCCAGG TGTGCCCCA ACCCAGC TGCCCCAGGCACTC TGCCCCCAG ACCCAGC TGCCCCAGGCACTC TGCCCCCAG ACCCAGC TGCCCCAGGCACTC TGCCCCCAG ACCCAGC TGCCCCAGGCACTC TCCACCACAC TGCCCCCAG AGGCACTCC TCCACCACAC TGCCCCCAGC ACCCAGC TGCCC ACCCAGC TGCCCCAGGCACTC TGCCCCCAGGACT TGCCCCCAG AGGCACTCC TGCCCCAGGCACTC TGCCCCCAGGCACTC TGCCCCCAGCACC TGCCCCAGCACC TGCCCCAGC		CACCTACAAC	ACAGACACGT	TTGAGTCCAT	GCCCAATCCC	GAGGGCCGGT	ATACATTCGG	CGCCAGCTGT	GTGAC	900
TRICCTGTCCC TACAACTACC TTTCTACGGA CGIGGGATCC TGCACCCTCG TCTGCCCCCT GCACAACCAA GAGGT TALACYSPTO TyrAsnTyrL CACACCAGAG GATGGAACA AGCGTGTGG GAGGACGCAA AGCGTGTGG GAGGACGCAA AGCGTGTGG GAGGACGCAA AGCGTGTGG GAGGACGCAA AGCGTGTGG GAGGACGCAA AGCGTGTGG CAGGACGTGA AGCGTGTGG CAGGACGTGA AGCGTGTGA CGAGGACGTGA AGCGTGTGA CGAGGACGTGA AGCGTGTGC CAGGACGTGA AGCGTGTGA CGAGGACGTGA AGCGTGTAC CAGGCCCCACC AGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTCA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACGTGA CGAGGACCTCC CAAGAACAT TTCTCCCCG AGACCTTCC AGACCTCTC TTCTCCCCG AGACCTTCA AGCGTTTCA TGCGGACCCCA CCCCCCCCT CCACCCAGAG CCCCCCCCC CCACCCAGAG CCCCCCCC										
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CCACCTCTGC TTCGTGCACA CGGTGCCCTG GGACCAGCTC TTTCGGAACC CGCACCAAGC TCTGCTCCAC ACTGC 1500 rHisLeuCys PheValHisT hrValProTr pAspGlnLeu PheArgAsnP roHisGlnAl aLeuLeuHis ThrAl CAACCGGCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGG 1575 aAsnArgPro GluAspGluC ysValGlyGl uGlyLeuAla CysHisGlnL euCysAlaAr gGlyHisCys TrpGl TCCAGGGCCC ACCCAGTGTG TCAACTGCAG CCAGTTCCTT CGGGGCCCAGG AGTGCGTGGA GGAATGCCGA GTACT 1650 yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800										
rHisLeuCys PheValHisT hrValProTr pAspGlnLeu PheArgAsnP roHisGlnAl aLeuLeuHis ThrAl CAACCGCCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGG 1575 aAsnArgPro GluAspGluC ysValGlyGl uGlyLeuAla CysHisGlnL euCysAlaAr gGlyHisCys TrpGl TCCAGGGCCC ACCCAGTGTG TCAACTGCAG CCAGTTCCTT CGGGGCCAGG AGTGCGTGGA GGAATGCCGA GTACT 1650 yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		CCACCTCTGC	TTCGTGCACA	CCCTCCCTC	GGACCAGCTC	TTTCGGAACC	CGCACCAAGC	TCTGCTCCAC	ACTGC	1500
CAACCGGCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGG 1575 aAsnArgPro GluAspGluC ysValGlyGl uGlyLeuAla CysHisGlnL euCysAlaAr gGlyHisCys TrpGl TCCAGGGCCC ACCCAGTGTG TCAACTGCAG CCAGTTCCTT CGGGGCCAGG AGTGCGTGGA GGAATGCCGA GTACT 1650 yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		rHisLeuCys	PheValHisT	hrValProTr	pAspGlnLeu	PheArgAsnP	roHisGlnAl	aLeuLeuHis	ThrAl	-
TCCAGGGCCC ACCCAGTGTG TCAACTGCAG CCAGTTCCTT CGGGGCCAGG AGTGCGTGGA GGAATGCCGA GTACT. 1650 yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		CAACCGGCCA	GAGGACGAGT	GTGTGGGCGA	GGGCCTGGCC	TGCCACCAGC	TGTGCGCCCG	AGGGCACTGC	TGGGG	1575
TCCAGGGCCC ACCCAGTGTG TCAACTGCAG CCAGTTCCTT CGGGGCCAGG AGTGCGTGGA GGAATGCCGA GTACT. 1650 yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		aAsnArgPro	GluAspGluC	ysValGlyGl	uGlyLeuAla	CysHisGlnL	euCysAlaAr	gGlyHisCys	TrpGl	
yProGlyPro ThrGlnCysV alAsnCysSe rGlnPheLeu ArgGlyGlnG luCysValGl uGluCysArg ValLe GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		TCCAGGGCCC .	ACCCAGTGTG	TCAACTGCAG	CCAGTTCCTT	CGGGGCCAGG	AGTGCGTGGA	GGAATGCCGA	GTACT.	1650
GCAGGGGCTC CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG AATGG 1725 uGlnGlyLeu ProArgGluT yrValasnal aargHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		yProGlyPro	ThrGlnCysV	alAsnCysSe	rGlnPheLeu	AraGlvGlnG	luCvsValGl	uGluCvsArg	ValLe	
uGlnGlyLeu ProArgGluT yrValAsnAl aArgHisCys LeuProCysH isProGluCy sGlnProGln AsnGl CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		GCAGGGGCTC	CCCAGGGAGT	ATGTGAATGC	CAGGCACTGT	TIGCCGTGCC	ACCCTGAGTG	TCAGCCCCAG	AATGG	1725
CTCAGTGACC TGTTTTGGAC CGGAGGCTGA CCAGTGTGTG GCCTGTGCCC ACTATAAGGA CCCTCCCTTC TGCGT 1800		uGlnGlyLeu	ProArgGluT	yrValAsnAl	aArgHisCys	LeuProCysH	isProGluCv	sGlnProGln	AsnGl	
ySerValThr CysPheGlyP roGluAlaAs pGlnCysVal AlaCysAlaH isTyrLysAs pProProPhe CysVa		CTCAGTGACC '	TGTTTTGGAC	CGGAGGCTGA	CCAGTGTGTG	GCCTGTGCCC .	ACTATAAGGA	CCCTCCCTTC	TGCGT	1800
		ySerValThr	CysPheGlyP	roGluAlaAs	pGlnCysVal	AlaCysAlaH	isTyrLysAs	pProProPhe	CysVa	

Fig. 8A

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GCCCCCTGC	CCCAGCGGTG	TGAAACCTGA	CCTCTCCTAC	ATGCCCATCT	GGAAGTTTCC	AGATGAGGAG	GGCGC	1875
IALBARGCYS	ProSerGlyV	alLysProAs	pLeuSerTvr	Met ProfleT	missephapr	oAenGluGlu	C1++8.1	
ATGCCAGCCT	' TGCCCCATCA	ACIGCACCCA	CICCIGIGIG	CACCTICCATIC	ACAAGGGGGGG	CCCCCCCAC	CACAC	1950
acyscinero	CysProlleA	snCvsThrHi	sSerCvsVal	Aspleuasna	entage@luCu	cProblaClu	Cl-3-	
WALCHARTCA	CIGALGICAL	TT-GAUGCACC	CGCCCCCCTCC	CCCAGCCCCA	CCACACACCC	CTCCCACCAT	CTVCNA	2025
galaserrro	LeuinrSerL	euGluAlaPr	OALaArgSer	ProSerProS	erThrClnDr	OTTOCIUNIC	Valaa	
TGCCATCCAG	GAGGCCCGGC	GICICCIGAA	CCTGAGTAGA	GACACTCCTC	CTCACATCAA	TEABACACTA	CAACE	2100
natatteGin	Glualaarga	rgLeuLeuAs	nLeuSerArg	AsoThrAlaA	laGluMetAs	nCluThrUal	Cluva	
CATCICAGAA	ATGITTGACC	TCCAGGAGCC	GACCTGCCTA	CAGACCCCCC	MACAL CALALY	CAACCACCCC	CITY OF	2175
IIIeserGiu	MetPheAspL	euGlnGluPr	oThrCvsLeu	GlnThrArd.	enGluLau?v	r[SetDav]v	I and a	
GGGLAGCCIC	ACCAAGCICA	AGGGCCCCTT	GACCATGATG	GCCAGCCACT	ACAAACAGCA	CALCALLEY	ACCCC	2250
geryserren	ThrLysLeuL	ysGlyProLe	uThrMetMet	AlaSerHisT	vr[vcG]nHi	eCreProPro	The De	2230
GGAAACTICC	TGTGCAACCC	AGATTATCAC	CTTTTGAAAGT	TTCAAACACA	ACCTICA ACCA	CALALANA CALALLA	CTICARO	2325
oGluThrSer	CysAlaThrG	lnIleIleTh	rPheGluSer	PhelysGluA	spi-pulvede	nPholouleu	Valti	2323
CCCCTTTGAC	TGCTGGGAGC	CAGTCCAGGA	GTGAGACCGG	CCAGATGAGG	CTCCCCAACC	princeabed	AGTII	2385
eProPheAsp	CysTrpGluP	roValGlnGl	u		CIGCCANGE			- 43 43

Fig. 8B

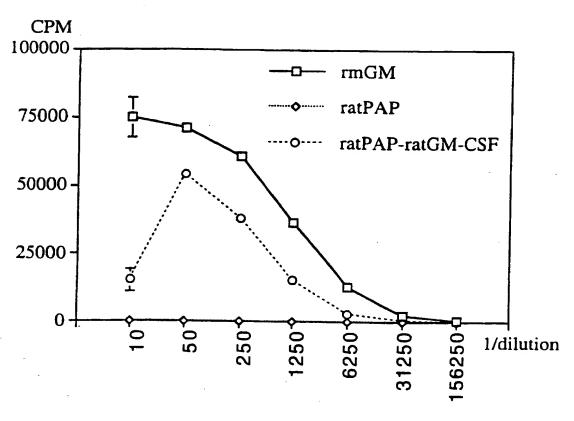


Fig. 9

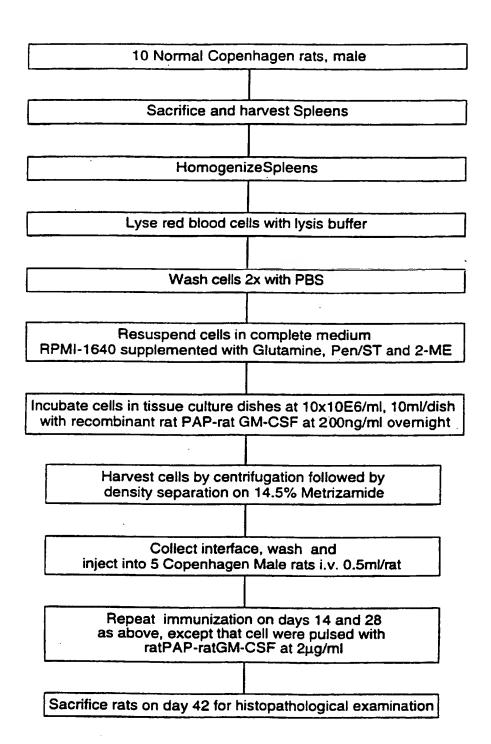


Fig. 10

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TCACTGCCAT GGAGGAGCCG CAGTCAGATC CTAGCGTCGA GCCCCCTCTG AGTCAGGAAA	60
Me tGluGluPro GinSerAspP roSerValGl uProProJeu SerGloGluT	\sim
CATTITICAGA CCIATOGAAA CIACITOCIG AAAACAACER TCTETTCCCC TTCCCCTCCC	120
nirrieseras pleuitplys LeuleuProg luAsnAsnVa lieuSerPro leuProCerc	120
AAGCAATICA TGATTIGATG CTGTCCCCGG ACCATATTIGA ACAATTGTTC ACTGAAGACC	100
Inalametas paspleumet LeuserProa spasnileci ucintrophe Throlyamp	180
CAGGICCAGA TGAAGCICCC AGAATGCCAG ACCTTCTTTC CTTTTTCTC CTTTTTCTC	240
rodlyproas poluatarro arometerros lualablada obrokalala broalabras	240
CARCITCIAL ACCUGUECO CCICCACCAC COCOMOTORIO COCOMOTORIO MOTORIORIO	300
laAlaProTh rProAlaAla ProAlaProA laProSerTr pProLeuSer SerSerValP	300
CTICCALAA AACCIACAG GGCAGCTACG GTTTTCCTTCTT COCTTTCTTTC CRITTCTCC	
roserGlnLy sthrtyrGln GlySerTyrG lyPheArgLe uGlyPheLeu HisSerGlyT	360
CAGCCAAGTC TGTGACTTGC ACGTACTCCC CTGCCCTCAA CAAGATGTTT TGCCAACTGG	
hrAlaLysSe rValThrCys ThrTyrSerP roAlaLeuAs nLysMetPhe CysGlnLeuA	420
CCAAGACCIG CCCTGIGCAG CIGIGCGITG ATTCCACACC CCCCCCGC ACCCGGICC	
laLysThrCy sProValGln LeuTrpValA spSerThrPr oProProGly ThrArgValA	480
GCGCCATGGC CATCTACAAG CAGTCACAGC ACATGACGGA GGTTGTGAGG CGCTGCCCCC	
rgAlaMetAl alleTyrLys GlnSerGlnH isMetThrGl uValValArg ArgCysProH	54 0
ACCATGAGGG CTGCTCAGAT AGCGATGGTC TGGCCCCTCC TCAGCATCTT ATCCGAGTGG	
isHisGluAr gCysSerAsp SerAspGlyL euAlaProPr oGlnHisLeu IleArgValG	60 0
AAGGAAATIT GCGIGIGGAG TATTIGGATG ACAGAAACAC TITICGACAT AGIGIGGGG	
luGlyAsnle uArgValGlu TyrLeuAspA spArgAsnTh rPheArgHis SerValValV	66 0
TOCCULATER GCOGCCIGAG GITGGCTCTG ACTGTACCAC CATCCACTAC AACTACATGT	
alProTyrGl uProProGlu ValGlySerA spCysThrTh rileHisTyr AsnTyrMetC	720
GTAACAGITC CIGCATGGGC GGATGAACC GGAGGCCCAT CCICACCATC ATCACACIGG	
VSASDSerSe nivementally Clambridge Control Control Attacking	780
ysAsnSerSe rCysMetGly GlyMetAsnA rgArgProIl eLeuThrIle IleThrLeuG	
AAGACTCCAG TOGTAATCTA CTGCGACCGA ACAGCTTTGA GGTGCGTGTT TGTGCCTGTC	840
luAspSerSe rGlyAsnLeu LeuGlyArgA snSerPheGl uValArgVal CysAlaCysP	
CTGGCAGAGA CCGGCGCACA GAGGAGAGA ATCTCCGCAA GAAAGGGGAG CCTCACCACG	900
roglyArgAs pargargThr GluGluGluA snLeuArgLy sLysGlyGlu ProHisHisG AGCTGCCCCC AGGGAGCACT AAGGGAGCAC TGCCCCAACAA CACCAGCTCC TCTCCCCAGC	
luleuProPr_cGlvSerThr_Lvm\nmalai	960
luLeuProPr oGlySerThr LysArgAlaL euProAsnAs nThrSerSer SerProGlnP CAAAGAAGAA ACCACIGGAT GGAGAATATT TCACCCTICA GATCCGIGGG CGIGAGCGCT	
Mississis epoletic Circles	1020
rolyslysly sProLeuAsp GlyGluTyrP heThrLeuGl nIleArgGly ArgGluArgP	
TOGAGATOTT COCAGACCTG AATGAGGCCT TOGAACTCAA GGATGCCCAG GCTGGGAAGG	1080
heGluMetPh eArgGluLeu AsnGluAlaL euGluLeuLy sAspAlaGln AlaGlyLysG	
ACCAGGGG GAGCAGGGCT CACTCCAGCC ACCTGAAGTC CAAAAAGGGT CAGTCTACCT	1140
luProGlyGl ySerArgAla HisSerSerH isLeuLysSe rLysLysGly GlnSerThrS	
CCCGCCATAA AAAACTCATG TTCAAGACAG AAGGGCCTGA CTCAGAGTCT AGGTCCGCAC	1200
erArgHisLy sLysLeuMet PheLysThrG luGlyProAs pSerAspSer ArgSerAlaP	
CCGCCCCCTC GCCCAGCCCC AGCACACAGC CCTGGGAGCA TGTGAATGCC ATCCAGGAGG	1260
roAlaArgSe rProSerPro SerThrGlnP roTrpGluHi sValAsnAla IleGlnGluA	
CCCGGCGTCT CCTGAACCTG AGTAGACACA CTGCTGCTGA GATGAATGAA ACAGTAGAAG	1320
laArgArgle uleuAsnleu SerArgAspT hrAlaAlaGl uMetAsnGlu ThrValGluV	
TCATCTCAGA AATGITICAC CTCCAGGAGC CGACCTGCCT ACAGACCCCC CTGCAGCTGT	1380
allleSerGl uMetPheAsp LeuGluGluP roThrCysLe uGlnThrArg LeuGluLeuT	
ACAAGCAGG CCTGCGGGC AGCCTCACCA AGCTCAAGGG CCCCTTGACC ATGATGGCCA	1440
yrlysglngl yleuArgGly SerleuThrL ysleuLysgl yProLeuThr MetMetAlas	
GCCACTACAA ACAGCACTGC CCTCCAACCC CGGAAACTTC CTGTGCAACC CAGATTATCA	1500
erHisTyrLy sGlnHisCys ProProThrP roGluThrSe rCysAlaThr GlnIlelleT	
CC111CECES 111CECES AACLICAACT ACTIVITY TO THE INTERPRETATION OF THE CONTRACT	1560
- ***FIEGIUSE IFIELVSCIU ASDIPIINEA CODNOTOUTO MEITO MEITO CONTOUR	
COCHRECARI CORRESPONDE ACTUAL ALL COLOR ALL CO	1610
rpGluProVa lGlnGluMet ArgGlySerH isHisHisHisHis	

Fig. 11

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MEEPQSDPSV	EPPLSQETFS	DLWKLLPENN	VLSPLPSQAM	DDLMLSPDDI	50
EQWFTEDPGP	DEAPRMPEAA	PPVAPAPAAP	TPAAPAPAPS	WPLSSSVPSQ	100
KTYQGSYGFR	LGFLHSGTAK	SVTCTYSPAL	NKMFCQLAKT	CPVQLWVDST	150
PPPGTRVRAM	AIYKQSQHMT	EVVRRCPHHE	RCSDSDGLAP	PQHLIRVEGN	200
LRVEYLDDRN	TFRHSVVVPY	EPPEVGSDCT	TIHYNYMCNS	SCMGGMNRRP	250
ILTIITLEDS	SGNLLGRNSF	EVRVCACPGR	DRRTEEENLR	KKGEPHHELP	300
PGSTKRALPN	NTSSSPQPKK	KPLDGEYFTL	QIRGRERFEM	FRELNEALEL	350
KDAQAGKEPG	GSRAHSSHLK	SKKGQSTSRH	KKLMFKTEGP	DSD <u>S R</u> SAPAR	400
SPSPSTQPWE	HVNAIQEARR	LLNLSRDTAA	EMNETVEVIS	EMFDLQEPTC	450
LQTRLELYKQ	GLRGSLTKLK	GPLTMMASHY	KQHCPPTPET	SCATQIITFE	500
FKENLKDFL	LVIPFDCWEP	VQEMRGSHHH	ннн		533

Fig. 12

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A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/12 C12N15/27 C12N15/55 C12N15/62 C12N9/16 C07K14/47 C07K14/71 A61K35/12 C12N5/08 CO7K14/535 According to International Patent Classification (IPC) or to both national classification and IPC **B. FIELDS SEARCHED** Minimum documentation searched (classification system followed by classification symbols) CO7K C12N A61K IPC 6 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) C. DOCUMENTS CONSIDERED TO BE RELEVANT Category ' Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No. 1 Х NATURE. vol. 344, 26 April 1990, pages 873-875, XP002031410 TAKAHASHI, H., ET AL .: "INDUCTION OF CD8+ CYTOTOXIC T CELLS BY IMMUNIZATION WITH PURIFIED HIV-1 ENVELOPE PROTEIN IN ISCOMs" see the whole document WO 95 23814 A (NAT JEWISH CENTER FOR 1 Х IMMUNOLO : KAPPLER JOHN W (US); MARRACK PHILI) 8 September 1995 see the whole document WO 94 21287 A (US HEALTH) 29 September 1,2 X 1994 see the whole document -/--Further documents are listed in the continuation of box C. Patent family members are listed in annex. " Special categories of cited documents: "T" later document published after the international filing date or priority date and not in conflict with the application but "A" document defining the general state of the art which is not considered to be of particular relevance ated to understand the principle or theory underlying the nognavai "E" earlier document but published on or after the international "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another involve an inventive step when the document is taken alone document of particular relevance; the claimed invention citation or other special reason (as specified) cannot be considered to involve an inventive step when the document is combined with one or more other such docu-"O" document referring to an oral disclosure, use, exhibition or other means ments, such combination being obvious to a person skilled document published prior to the international filing date but later than the priority date claimed '&' document member of the same patent family Date of mailing of the international search report Date of the actual completion of the international search D 3. 06. 97 27 May 1997 Name and mailing address of the ISA Authorized officer European Patent Office, P.B. 5818 Patentiaan 2 NL - 2280 HV Risswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 Holtorf, S

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PCT/US 96/20241

	non) DOCUMENTS CONSIDERED TO BE RELEVANT	Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Retvant & Claim 140.
x	NATURE, vol. 362, 22 April 1993, pages 755-758, XP002031411 TAO,MH., ET AL.: "IDIOTYPE/GRANULOCYTE - MACROPHAGE COLONY-STIMULATION FACTOR FUSION PROTEIN AS A VACCINE FOR B-CELL LYMPHOMA" see the whole document	15,16, 18,25,30
P,X	WO 96 37107 A (SCRIPPS RESEARCH INST) 28 November 1996 see the whole document	1
P,X	VACCINE, vol. 14, no. 3, 1996, pages 230-236, XP002031412 TARPEY, I., ET AL .: "PRIMING IN VIVO AND QUANTIFICATION IN VITRO OF CLASS I MHC-RESTRICTED CYTOTOXIC T CELLS TO HUMAN PAPILLOMA VIRUS TYPE 11 EARLY PROTEINS (E6 AND E7) USING IMMUNOSTIMULATING COMPLEXES (ISCOMs)" see the whole document	1
P,X	WO 96 22377 A (ARMITAGE IAN M ;SANDLIE INGER (NO); BOGEN BJARNE (NO)) 25 July 1996 see the whole document	1,2,7,8, 11,12, 15,18, 20,22, 23,25,30
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A	WO 95 21862 A (BRIGHAM & WOMENS HOSPITAL) 17 August 1995 see the whole document	1-30

ternational application No.

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of lifst sheet)
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X Claims Nos.: 13,14 because they relate to subject matter not required to be searched by this Authority, namely: Remark: Although claims 13,14 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This International Searching Authority found multiple inventions in this international application, as follows:
1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark on Protest The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:
C12N 15/12, 15/27, 15/55, 15/62, 9/16, 5/08, C07K 14/535, 14/47, 14/71, A61K

A1

(11) International Publication Number:

WO 97/24438

(43) International Publication Date:

10 July 1997 (10.07.97)

(21) International Application Number:

PCT/US96/20241

(22) International Filing Date:

23 December 1996 (23.12.96)

(30) Priority Data:

35/12

08/579,823

28 December 1995 (28.12.95) US

(74) Agents: STRATFORD, Carol, A. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

(60) Parent Application or Grant

(63) Related by Continuation

Filed on

08/579,823 (CIP) 28 December 1995 (28.12.95)

(71) Applicant (for all designated States except US): ACTIVATED CELL THERAPY, INC. [US/US]: 291 North Bernardo Avenue, Mountain View, CA 94043 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): LAUS, Reiner [DE/US]; 2813 Porto Rosa Way, San Carlos, CA 94070 (US). RUEGG, Curtis, Landon [US/US]; 3358 Brittan Avenue, San Carlos, CA 94070 (US). WU, Hongyu [US/US]; 275 Ventura Avenue #19, Palo Alto, CA 94306 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.
With amended claims and statement.

Date of publication of the amended claims and statement:

4 September 1997 (04.09.97)

(54) Title: IMMUNOSTIMULATORY COMPOSITION AND METHOD

(57) Abstract

Disclosed are therapeutic compositions and methods for inducing cytotoxic T cell responses in vitro and in vivo. The therapeutic compositions consist of antigen presenting cells activated by contact with a polypeptide complex constructed by joining together a dendritic cell-binding protein and a polypeptide antigen. Also disclosed are expression vectors and systems for producing the polypeptide complexes.

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AMENDED CLAIMS

[received by the International Bureau on 15 July 1997 (15.07.97); original claims 1-30 replaced by amended claims 1-29 (4 pages)]

- 1. A therapeutic composition, comprising an isolated potent antigen presenting cell which is stimulated by exposure *in vitro* to a polypeptide complex consisting of a dendritic cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene gene product, wherein said composition is effective to activate T-cells to produce a multivalent cellular immune response against a selected antigen, at a T-cell activation level substantially higher than a level produced by said antigen presenting cells stimulated by the selected antigen alone.
- 2. The therapeutic composition of claim 1, wherein the dendritic cell-binding protein is GM-CSF.
 - 3. The therapeutic composition of claim 1, wherein the polypeptide antigen is the tumor-specific antigen prostatic acid phosphatase.
- 4. The therapeutic composition of claim 1, wherein the polypeptide antigen is an oncogene product peptide antigen selected from the group consisting of Her2, p21RAS, and p53.
- 5. The therapeutic composition of claim 1, wherein the polypeptide complex further comprises, between said dendritic cell-binding protein and said polypeptide antigen, a linker peptide.
 - 6. The therapeutic composition of claim 1, wherein the potent antigen presenting cell is an activated dendritic cell.
 - 7. A method of activating an isolated antigen presenting cell in vitro, comprising

contacting said isolated antigen presenting cell with a polypeptide complex consisting essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen,

wherein said activated antigen presenting cell is effective to activate a T-cell to produce a multivalent cellular immune response that is substantially higher than that produced by antigen presenting cells contacted with the selected polypeptide antigen alone.

- 8. The method of claim 7, wherein said dendritic cell-binding protein is GM-CSF.
- 9. The method of claim 7, wherein said polypeptide antigen is the tissue-5 specific tumor antigen prostatic acid phosphatase.
 - 10. The method of claim 7, wherein the polypeptide complex is a fusion protein.
- 10 11. The method of claim 7, wherein said isolated antigen presenting cell is a dendritic cell.
 - 12. A method of inducing a cytotoxic T-cell response in a vertebrate subject, comprising
- contacting an isolated antigen presenting cell with a polypeptide complex comprising a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen for a period of time effective to activate said antigen presenting cell, and
- 20 injecting said antigen presenting cell into the mammalian subject.
 - 13. The method of claim 12, wherein said antigen presenting cell is a dendritic cell.
- 25 14. A polypeptide complex, comprising an isolated tissue-specific tumor antigen covalently linked to a dendritic cell-binding protein.
 - 15. The polypeptide complex of claim 14, wherein said dendritic cell-binding protein is GM-CSF.
- The polypeptide complex of claim 14, wherein said tissue-specific tumor antigen is prostatic acid phosphatase.
- 17. The polypeptide complex of claim 14, wherein the complex is a fusion protein.
 - 18. The polypeptide fusion protein complex of claim 14, which further includes, between said dendritic cell binding protein and said tissue-specific tumor antigen, a linker peptide.

- 19. A polypeptide complex, comprising an isolated oncogene product peptide antigen covalently linked to a dendritic cell-binding protein.
- 20. The polypeptide complex of claim 19, wherein said dendritic cell-binding protein is GM-CSF.
 - 21. The polypeptide complex of claim 19, wherein said oncogene product peptide antigen is selected from the group consisting of Her2, p21RAS, and p53.
- The polypeptide complex of claim 19, wherein the complex is a fusion protein.
- 23. The polypeptide fusion protein of claim 19, which further includes, between said dendritic cell binding protein and said oncogene product antigen, a linker peptide.
- 24. An expression vector for producing an immunostimulatory fusion protein, comprising a nucleic acid molecule encoding a polypeptide complex consisting essentially of a dendritic cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen, and an oncogene product peptide antigen, said nucleic acid molecule inserted into an expression vector, wherein said nucleic acid molecule is operably linked to a selected promoter able to initiate transcription in a selected host cell.
 - 25. The expression vector of claim 24, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the tissue-specific tumor antigen prostatic acid phosphatase.
 - 26. The expression vector of claim 24, wherein said dendritic cell-binding protein is GM-CSF and said polypeptide antigen is the oncogene product peptide antigen Her2.
- 35 27. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and prostatic acid phosphatase.
 - 28. A substantially purified nucleic acid molecule encoding a fusion protein consisting essentially of GM-CSF and Her2.

29. An expression system for producing a fusion protein consisting essentially of a dendritic cell-binding protein covalently linked to a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene product peptide antigen, comprising

a nucleic acid sequence encoding a dendritic cell-binding protein,

a nucleic acid sequence encoding the polypeptide antigen, each of said nucleic acid sequences inserted into an expression vector, wherein said nucleic acid sequences are operably linked to a promoter able to initiate transcription in a selected host cell, and

said expression vector is carried within the host cell.

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STATEMENT UNDER ARTICLE 19

Claim 1 has been amended to include the limitations previously present in Claim 2, now deleted by this amendment. The remaining claims have been renumbered accordingly. This amendment brings out the feature that the potent antigen presenting cells that form the main composition of the invention are stimulated *in vitro* with a polypeptide complex consisting of a dendritic cell-binding protein and a polypeptide antigen selected from the group consisting of a tissue-specific tumor antigen and an oncogene gene product. This distinguishes the cell composition from those cells described in the cited prior art, which generally involve *in vivo* stimulation of cells (e.g., Takahashi, et al., Nature 344: 873; Tao and Levy, Nature 362: 755; WO 95/23814). The amendment also distinguishes the invention over WO 95/21287, which is concerned with dendritic cells coated with peptides, rather than with polypeptide complexes.

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